THE

GENETIC BASIS

OF

HEMIFACIAL MICROSOMIA

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Abstract

The aetiology of hemifacial microsomia is uncertain; vascular, metabolic, teratogenic and genetic factors have been proposed as aetiological influences. The evidence for a genetic basis comes from observations of the phenotype in individuals carrying chromosomal rearrangements, and from a small number of studies involving families in whom the condition appeared to be transmitted in a Mendelian fashion. Malformations reminiscent of hemifacial microsomia have also been seen in knockout and mutant animal models, and these have given rise to a number of candidate genes for the disease.

Two main objectives were defined at the start of this project. The first of these was to expand upon the currently available clinical data by interviewing and examining patients with hemifacial microsomia or isolated microtia who presented to two specialist treatment centres in London, Great Ormond Street Hospital and Mount Vernon Hospital. Alongside this a genome scan was performed on a family in which hemifacial microsomia appeared to be segregating in an autosomal dominant manner. Whilst definite linkage to a single locus could not be inferred from the results of the genome scan, a few regions of interest were identified, and linkage to a large proportion of the genome was excluded. One area of particular interest, 2q32 – 2q37, was analysed in greater detail, and other regions together with their potential disease genes have been highlighted.

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1. Introduction

The term hemifacial microsomia has been used to describe a range of abnormalities that results from the abnormal development of anatomical structures which are derived from the first and second branchial arches, and it is one of the most common craniofacial malformations observed in humans.

The disorder includes a wide spectrum of anomalies primarily affecting the external and middle ear, the mandible and the maxilla; therefore, patients with hemifacial microsomia are managed by a number of different medical specialties e.g. ENT surgery, plastic surgery, maxillo-facial surgery, ophthalmology, and clinical genetics.
Some children are born with microtic ears, but have none of the other features associated with hemifacial microsomia, and debate continues as to whether these cases of isolated microtia constitute an aetiologically distinct condition or a mild form of hemifacial microsomia.

The aim of this work is to investigate the genetic basis of hemifacial microsomia.

1.1 Incidence & Epidemiology

Many patterns of craniofacial malformation have been observed in man, and amongst these hemifacial microsomia is relatively common with only the cleft lip and palate deformities having a higher reported incidence. The incidence of hemifacial microsomia is most often quoted as 1/5600 births {Grabb, 1965 46 /id}. This figure was determined by a retrospective review of the records of 39,492 births in Michigan, USA, amongst whom there were 7 infants with hemifacial microsomia. Stoll et al {Stoll, 1984 60 /id} quoted an incidence of 1/19,500 consecutive births in France, Melnick {Melnick, 1980 5 /id} reported a frequency of 1/26,550 live births in a prospective study in America, and Higurashi et al {Higurashi, 1990 157 /id} reported a single case of Goldenhar syndrome in 27,472 consecutive births in Tokyo, Japan. The variations in prevalence quoted are likely to be due, at least in part, to differences in the inclusion criteria used by different research groups, and thus their ascertainment of cases, although true geographical variation cannot be discounted.

The prevalence of isolated microtia also varies greatly between different reports. Mastroiacovo et al {Mastroiacovo, 1995 105 /id} reported a rate of 1.46/10 000 using data collected from the Italian Multicentre Birth Defects Registry. A report from South America {Castilla, 1986 62 /id} suggested a prevalence of 17.4/10,000. Data from three registries of congenital malformations in France, Sweden and America were examined by Harris, Källén and Robert {Harris, 1996 175 /id}, who found prevalence rates for isolated microtia/anotia of 0.57, 1.58 and 1.01 per 10,000 in each of these countries

respectively. A significant difference in prevalence rates between racial groups was noted within the group of patients from California, USA, included in this analysis; the prevalence was highest in Hispanics (3.23/10,000) and Asians (2.18/10,000), and lower in blacks and whites, 1.22 and 1.17 per 10,000 respectively. These differences suggest a genetic component to the aetiology of microtia as they are unlikely to be due to variations in inclusion criteria or case ascertainment, both of which are important considerations when comparing figures from different registries.

The International Clearinghouse for Birth Defects Monitoring Systems records data submitted voluntarily from 31 participating programmes around the world, with cases of anotia and microtia being amongst the birth defects monitored. Within this database the rate of anotia and microtia per 10 000 births for the year 2000 ranged from 20.85 (Canada: British Columbia) to 0 (Dublin, Malta, North Netherlands, United Arab Emirates), though most rates are close to the mean of 2.55/10 000 {ICBDMS, 2002 61 /id}. The incidence of anotia in England and Wales in 2000 as reported to the ICBDMS was 0.13/10 000 (8 cases in 604,130 births), and 3 cases of microtia were documented, which was equivalent to an incidence of 0.05/10 000. However, the differentiation between anotia and severe microtia is imprecise; in our personal experience in the microtia clinic at Great Ormond Street Hospital we have already recorded five patients who were born in 2000 who we classified as having isolated microtia, but we have not seen any cases that we would consider to be anotia.

1.2 Clinical features

The clinical phenotype of hemifacial microsomia is highly variable. Although no minimal diagnostic criteria have been agreed upon, the principle features of the defect are those of asymmetric facial and auricular abnormalities.

1.2.1 Facial abnormalities

The facial asymmetry that is a feature of hemifacial microsomia may result from a deficiency of both the skeletal and soft tissue components of the facial structures that are derived from the first branchial arch. Hence, the temporomandibular joint, mandible, maxilla, and muscles of mastication may all be hypoplastic. Facial asymmetry may not be readily apparent at birth, but differential growth of the normal and affected structures will produce an evident deformity by the age of four {Rollnick, 1987 2 /id} {Kearns, 2000 163 /id} {Gorlin 2002 49 /id}. In cases of unilateral facial involvement, several reports suggest that the right side of the face is more commonly affected than the left; Rollnick and Kaye reported that 40% of patients showed right sided laterality in their series of 202 patients with mandibular hypoplasia and microtia, compared to 28% with the opposite laterality {Rollnick, 1987 2 /id}. Bilateral facial involvement occurs in 6-31% of cases with one side of the face being more severely affected than the other in almost all patients {Grabb, 1965 46 /id} {Rollnick, 1987 2 /id}.

Macrostomia is sometimes present and is almost always unilateral and ipsilateral to the more affected side of the face. Cleft lip and/or palate occurs in up to 19% of patients either unilaterally or bilaterally {Feingold, 1978 16 /id} {Rollnick, 1987 2 /id}.

1.2.2 Ear abnormalities

The abnormalities of the external ear that are seen in patients with hemifacial microsomia range from pinnae that are normally shaped but abnormally small, to cases in which the external ear is represented by a small, ill-defined mass of tissue located antero-inferiorly to the normal site of the pinna, and to anotia in the most extreme cases. A number of grading systems classifying the different degrees of microtia have been proposed {Rollnick, 1987 2 /id} {Meurman, 1957 102 /id} {Ogino, 1979 140 /id} {Jani, 1998 150 /id}. The grade of microtia may provide an indication of the degree of middle ear maldevelopment in patients with aural atresia {Kountakis, 1995 106 /id}, but apart from this, the grading of microtia has limited clinical importance since the microtic deformity in hemifacial microsomia is not a progressive condition and the management of microtia is highly dependent upon the individual patient's subjective need for treatment, and less dependent on the precise anatomical deformity.

The deformities required to define an individual as having hemifacial microsomia vary between different authors, and this renders it difficult to assign a frequency for the occurrence of any particular deformity in hemifacial microsomia. This is particularly

true of microtia as some authors have used microtia as their minimal diagnostic criterion for the inclusion of individuals into studies of hemifacial microsomia, whilst others would not exclude the diagnosis of hemifacial microsomia in the absence of a deformity of the external ear {Ardingen, 1988 197 /id}. One of the papers often cited in respect of this controversy is that written by Bennun et al {Bennun, 1985 50 /id} in which the argument is made that microtia is a microform of hemifacial microsomia on the basis of similarities between the two conditions with regards to the incidence and pattern of facial nerve weakness and hemipalatal palsy, the presentation of asymmetry, and a male prevalence, together with a knowledge of the embryological origins of the ear and face. Bennun reviewed the data on 74 patients with isolated microtia. The series contained a 2:1 male to female preponderance, and a similar unequal sex distribution had been noted in other reports of hemifacial microsomia patients {Grabb, 1965 46 /id} {Rollnick, 1983 57 /id}. Twelve percent of Bennun's patients had facial nerve weakness, which was always ipsilateral to a severe microtia. The incidence of facial palsy in hemifacial microsomia was also high in another report {Murray, 1984 83 /id}. Hemipalatal palsy was seen in both microtia and hemifacial microsomia and it has been suggested that this weakness is due to defective innervation of the levator veli palatini muscle by a proximal branch of the facial nerve. Asymmetry was common in both microtia and hemifacial microsomia; both conditions usually occurring in a unilateral form, and when bilateral involvement occurred it was always asymmetrical. The right side of the head was affected in 57% of cases of microtia and in 56% of hemifacial microsomia patients.

At present the relationship of isolated microtia to hemifacial microsomia remains undefined, but elucidation of the genetic basis of hemifacial microsomia would allow resolution of this uncertainty.

Stenosis or atresia of the external auditory canal is sometimes found in association with anomalies of the pinna. A conductive hearing loss may be caused by atresia of the ear canal and also by malformation of the ossicular chain.

1.2.3 Central nervous system abnormalities

A range of central nervous system defects has been reported in association with hemifacial microsomia. The most common neurological defect is a facial nerve palsy that is seen in 10-22% {Carvalho, 1999 51 /id} and which is likely to be due to distortion of the bony facial nerve canal. In a series of 40 patients with hemifacial microsomia, temporal bone computed tomographic scanning revealed abnormalities of the facial nerve canal in 35 patients (88%) {Rahbar, 2001 86 /id}. The facial nerve was said to be anteriorly displaced in 25 patients, and could not be identified in 10 patients.

Mental retardation is estimated to occur in 5-15% of patients with hemifacial microsomia {Shokeir, 1977 152 /id}.

1.2.4 Eye abnormalities

Choristomas, which are tumours composed of normal tissue not normally found at the site of the tumour, are the most common ocular abnormality in hemifacial microsomia. They contain a combination of fat, hair follicles and sebaceous cysts, and they are sometimes divided into dermoids and lipodermoids depending on the amount of fat they contain. Mansour et al {Mansour, 1985 84 /id} noted epibulbar choristomas in 32% of a series of 57 consecutive patients with facioauriculovertebral sequence. An earlier review of 127 patients compiled from a number of published series of Goldenhar's syndrome found that 53% had unilateral epibulbar dermoids and 23% had bilateral dermoids {Baum, 1973 85 /id}. That review also found an incidence of 47% for lipodermoids, but there was, no doubt, a degree of bias towards ocular abnormalities in the cases reviewed since epibulbar dermoids are one of the defining features of Goldenhar's syndrome {Goldenhar, 1952 155 /id}.

Other eye signs associated with hemifacial microsomia include eyelid colobomas, ocular motility disorders, anophthalmia and microphthalmia {Baum, 1973 85 /id}.

1.2.5 Skeletal abnormalities

Cervical spine and skull base abnormalities occur as cervical vertebral fusions (20%-35%), Klippel-Feil syndrome, platybasia and occipitalization of the atlas {Avon, 1988 199 /id}. Vertebral anomalies such as spina bifida, hemivertebrae, butterfly vertebrae,

vertebral fusion, vertebral hypoplasia, and scoliosis occur in 30% of patients. Talipes equinovarus has been reported in about 20% of patients. Radial limb deformities in the form of a hypoplastic or aplastic radius and/or thumb, or bifid or digitalised thumb, occur in about 10% {Gorlin, 1963 109 /id}.

1.2.6 Other abnormalities

Various forms of cardiac anomaly, most commonly ventricular septal defects and tetralogy of Fallot, have been reported in individuals with hemifacial microsomia. Cardiovascular abnormalities such as these and anomalies of the great vessels are present in between 5% and 30% of cases {Pierpont, 1982 177 /id} {Rollnick, 1987 2 /id} {Morrison, 1992 156 /id}.

Other congenital defects have occurred occasionally on a background of hemifacial microsomia, but in view of the limited number of reports of each combination of defects it is uncertain whether these represent true associations or merely coincidental findings.

Occasional reports have been made of cranial nerve palsies other than facial nerve weakness, and of intracranial anomalies including occipital and frontal encephalocoeles, hydrocephaly, lipoma of the corpus callosum, dermoid cyst, teratoma, Arnold-Chiari malformation, lissencephaly, arachnoid cyst, holoprosencephaly, porencephalic cyst,

unilateral arrhinencephaly, and hypoplasia of the corpus callosum {Aleksic, 1975 87 /id} {Aleksic, 1984 200 /id}.

Bronchopulmonary defects that have been reported in patients with hemifacial microsomia and these include incomplete lobulation, hypoplasia and agenesis, and tracheo-oesophageal fistulae {Sutphen, 1995 176 /id}.

Renal involvement may be manifested as renal agenesis, duplex systems, hydronephrosis, and renovascular anomalies {Bowen, 1971 88 /id} {Shokeir, 1977 152 /id} {Rollnick, 1987 2 /id}. Imperforate anus with or without rectovaginal fistula has also been described in association with hemifacial microsomia {Bowen, 1971 88 /id}.

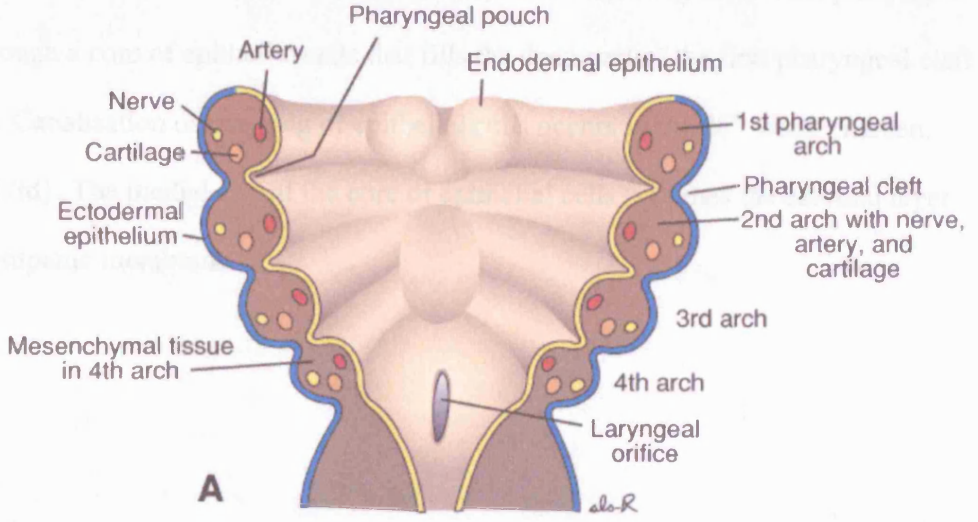
1.3 Nomenclature

As a result of the variable clinical presentation seen in these patients, and the lack of an agreed diagnostic standard, a number of different terms have been coined that probably identify variants of the same spectrum of anomalies; hence, oculo-auriculo-vertebral dysplasia, oculo-auriculo-vertebral spectrum, Goldenhar-Gorlin syndrome, first arch syndrome, first and second branchial arch syndrome, lateral facial dysplasia, and a number of other terms have all been recorded in the literature. The combination of facial asymmetry, microtia and co-existent eye anomalies, particularly epibulbar dermoids, and vertebral anomalies usually acquires the eponymous label of Goldenhar syndrome, though Goldenhar concentrated his report on the triad of epibulbar dermoids, auricular appendages and preauricular fistulae {Goldenhar, 1952 155 /id}. The characteristic features assigned to each of these appellations differ between authors and this is, no doubt, a source of ascertainment bias between individual series. The proliferation of terms attests to the fact that none is obviously superior when attempting to encompass the large number of different phenotypes observed. The nosologic difficulties are compounded because of the likely heterogeneous aetiology and pathogenesis of this group of malformations. I have chosen to use the term hemifacial microsomia to encompass all of the above conditions in this thesis.

1.4 Embryology

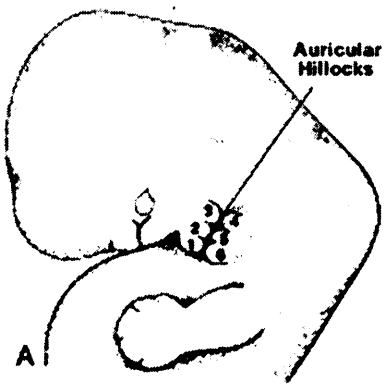
The structures of the face and ear that are abnormal in hemifacial microsomia are derived from the first and second branchial arches in the embryo. During the fourth post conceptual week segmentation of the mesenchyme of the ventral foregut region produces five distinct bilateral swellings called the pharyngeal, or branchial, arches. Each arch has a core of mesoderm surrounded by neural crest tissue that has migrated ventrally from the hindbrain. The neural crest cells give rise to skeletal and connective tissues, whilst the muscular tissue is derived from the mesoderm. Externally the branchial arches are separated by the pharyngeal clefts or grooves, and there are five corresponding internal sulci termed the pharyngeal pouches (figure 1).

Figure 1. Schematic drawing of the pharyngeal arches. (from {Sadler, 1985 202 /id})



The external ear (auricle or pinna) is derived from six auricular hillocks (figure 2) that develop at the caudal end of the first and second branchial arches{O'Rahilly, 1996 47 /id}. The hillocks initially appear at 5 weeks and soon fuse to form the auricle.

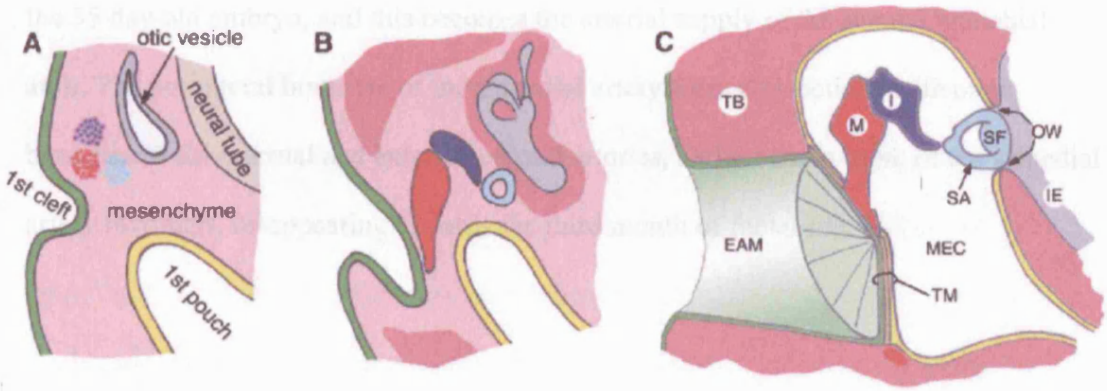
Figure 2. Lateral view of the head of an embryo showing the six auricular hillocks (from {Sadler, 1985 202 /id})



The external acoustic meatus (ear canal) develops via deepening of the first pharyngeal cleft through a core of epithelial cells that fills the deep part of the first pharyngeal cleft initially. Canalisation of this plug of epithelial cells occurs in the 26th week {Larsen, 1993 48 /id}. The medial end of the core of epithelial cells becomes the external layer of the tympanic membrane.

The first pharyngeal pouch elongates to form the tubotympanic recess, which then differentiates into the tympanic cavity and the eustachian tube. As the developing ectodermal external auditory meatus and the endodermal tubotympanic recess approach one another the auditory ossicles arise from the intervening mesenchyme. During the seventh week the three ossicles condense from the mesenchyme of the first and second arches, the first arch gives rise to the incus and the head of the malleus, and the second arch to the stapes and the long process of the malleus. The ossicles remain embedded in mesenchyme until the ninth month when the mesenchyme disappears and the tympanic cavity enlarges to include them (figure 3).

Figure 3: Schematic representation of the development of the middle ear structures.

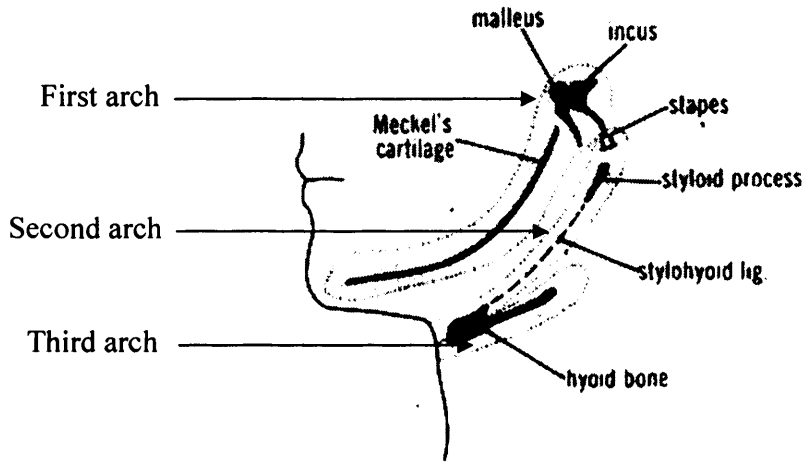


A: 5 week old embryo. B:7 week old embryo. C: Fully developed middle ear. EAM=external auditory meatus, TB=temporal bone, M=malleus, I=incus, SA=stapes arch, SF=stapes footplate, OW=oval window, IE=inner ear, TM=tympanic membrane, MEC=middle ear cavity. (from {Mallo, 2001 201 /id})

The first arch also gives rise to the bony structures of the mandibular and maxillary regions of the face, the muscles of mastication, the anterior belly of digastric, the mylohyoid, tensor tympani and tensor palatini muscles. The motor innervation to the muscles derived from the first pharyngeal arch is from the mandibular branch of the trigeminal nerve. The blood supply to the first pharyngeal arch comes via the maxillary artery.

The second arch cartilage, Reichert's cartilage, as well as contributing the stapes gives rise to the styloid process of the temporal bone, the stylohyoid ligament and the superior portion of the hyoid bone (figure 4). The muscles of the second branchial arch are stapedius, stylohyoid, the posterior belly of digastric, the auricular muscles, and the muscles of facial expression, and these muscles are innervated by the facial nerve. A small branch arises from the second and first aortic arches to form the stapedial artery in the 35 day old embryo, and this becomes the arterial supply of the second branchial arch. The peripheral branches of the stapedial artery form connections with other branches of the internal and external carotid arteries, and the main trunk of the stapedial artery involutes, disappearing towards the third month of foetal life.

Figure 4: Schematic representation of the derivatives of the first three branchial arches (modified from {Sadler, 1985 202 /id})



Thus, a disruption of the normal progress of development of the first and second branchial arch tissues could be expected to cause malformations of the pinna, the auditory meatus, the ossicles, and the bony and soft tissue elements of the maxillary and mandibular regions of the face, and might also be associated with a seventh cranial nerve defect that would present as a facial palsy. These malformations are typical of those found in patients with hemifacial microsomia.

1.5 Aetiology

It is likely that the aetiology of hemifacial microsomia is multifactorial, and it is possible that hemifacial microsomia is the result of the interplay between several genetic and environmental influences. Several theories about the causal mechanisms underlying hemifacial microsomia have been proposed and these include vascular, metabolic, teratogenic and genetic factors.

1.5.1 Vascular

Poswillo created a phenocopy of hemifacial microsomia in an animal model in which an expanding haematoma in utero damaged tissues in the region of the ear and jaw {Poswillo, 1973 59 /id}. Administration of triazene diluted in arachis oil to mice on day 10.5 of gestation produced 100% affected offspring with the characteristic defects of hemifacial microsomia. Serial studies of the developing vascular tree showed that the haematoma originated at the junction of the ascending pharyngeal and hyoid arteries, the site of the primitive stem of the stapedial artery {Poswillo, 1975 8 /id}. The timing of the commencement of proliferation of the stapedial artery on day 14.5 coincided with the appearance of haemorrhage in this region. Poswillo observed a range of phenotypes with variable involvement of the outer and middle ear, mandible, and facial soft tissues. Poswillo proposed that the obstructive and destructive effects of an extravascular blood clot would result in focal cell death, repair and redifferentiation, producing the observed

malformations. Variable degrees of haematoma formation would account for the spectrum of extent and severity found in hemifacial microsomia. However, the factors which led to the onset of haemorrhage were not apparent.

Werler et al {Werler, 2004 181 /id} interviewed the mothers of 230 patients with hemifacial microsomia, and compared them to controls matched for age and paediatric practice. The multivariate odds ratios were significantly increased for exposure to pseudoephedrine (2.0, 95% CI 1.2-3.4), and for exposure to any of the following vasoactive medications, pseudoephedrine, phenylpropanolamine, aspirin or ibuprofen (1.9, 95% CI 1.2-2.9).

1.5.2 Metabolic

Maternal diabetes is well known to have teratogenic effects and has been linked to anomalies such as caudal dysgenesis, congenital heart defects, renal anomalies and neural tube defects. Craniofacial anomalies such as cleft lip and palate have been reported occasionally in infants born to diabetic mothers. The malformations that are characteristic of hemifacial microsomia have also been reported in infants of diabetic mothers {Grix, 1982 78 /id}{Johnson, 1982 168 /id}{Ewart-Toland, 2000 79 /id}. Since the association is not a common finding a number of other factors such as an underlying genetic predisposition must be involved if there is a true link between the two conditions.

Wang et al {Wang, 2002 189 /id} analysed data from a congenital birth defects registry in Spain that surveyed a total population of 1,731,791 consecutive live-born infants, and which included 157 cases of oculo-auriculo-vertebral sequence. The data suggested that the odds ratio for oculo-auriculo-vertebral sequence in infants of mothers with gestational diabetes mellitus was 2.28 (95% CI, 1.03-4.82). A greater risk of hemifacial microsomia in relation to maternal diabetes was calculated by Werler et al {Werler, 2004 181 /id}. They calculated a multivariate odds ratio of 6.0 (95% CI 2.5-14.3).

The mechanism by which maternal diabetes produces malformations has not been defined, but hypotheses include hyperglycaemic damage to vessels, or mutagenesis via the production of excess reactive oxygen species {Reece, 1996 182 /id}.

1.5.3 Teratogenic

The exposure of pregnant women to certain environmental agents, termed teratogens, may produce malformations in their offspring. The drug thalidomide was administered as an anti-nausea treatment to pregnant women from 1958 to 1961. The children of these mothers were born with facial anomalies that included anotia, microtia, meatal stenosis and atresia, ear tags, facial palsy, and eye abnormalities; other features of thalidomide exposure were limb defects, cleft palate, choanal atresia, cardiac defects and oesophageal or duodenal atresia {Gorlin, 2001 148 /id}. Poswillo reported studies

which showed that the otomandibular defects produced by thalidomide in Macaca irus monkeys were closely comparable to those found in thalidomide embryopathy in man {Poswillo, 1975 8 /id}, and that the malformations recorded resembled the features of hemifacial microsomia. Examination of thalidomide-affected embryos revealed haematomas involving the tissues of the outer and middle ear and mandible. Thus, Poswillo proposed that haemorrhage and self-limiting haematoma formation were the common causal mechanism for the phenotypes noted in both sporadic and thalidomide induced hemifacial microsomia, though the factors leading to the onset of haemorrhage remained unclear.

Some features of the hemifacial microsomia phenotype have been associated with the exposure of pregnant women to other teratogens, namely primidone {Gustavson, 1985 76 /id}, retinoic acid {Lammer, 1985 77 /id}, tamoxifen {Cullins, 1994 158 /id}, and cocaine {Lessick, 1991 169 /id}.

Thus, vascular, metabolic and teratogenic factors have all been implicated in the aetiology of hemifacial microsomia, and each may interact with genetic influences in a particular affected individual.

1.5.4 Multiple gestations

The frequency of embryonic malformations is increased in twins compared to singleton gestations, and this excess is due to an increased incidence of malformations in monozygotic twins alone {Schinzel, 1979 89 /id}. There are three potential causes for the increased liability of twins to congenital malformations. The first of these is that the underlying cause of twinning could also cause malformations. Secondly, most monozygotic twins have a conjoined placenta producing vascular connections between the two foetuses; unequal vascular interchange between the twins could result in a reduced perfusion pressure in one twin and resultant disruption of inadequately perfused tissues. Also, following the death of one monozygotic twin in utero, clots or detritus from the dead twin may enter the circulation of the live twin and form an embolus, leading to the development of malformations in the surviving co-twin. Thirdly, the presence of two foetuses within the constrained limits of the uterus can produce structural deformations. It is unlikely that this last factor is important in the aetiology of hemifacial microsomia since the defects such as complete anotia, auditory meatal atresia, and facial palsy, which are found in hemifacial microsomia suggest an intrinsically abnormal developmental process rather than the effects of external mechanical forces.

Lawson et al {Lawson, 2002 90 /id} found an increased prevalence of twin maternities amongst 145 cases of microtia and hemifacial microsomia (3.96%, 95% CI 0.79 to 7.13) compared to the age standardised prevalence rates for England and Wales (1.06%). The report by Lawson et al included some information from the database of patients that was collected as part of the work on which this thesis is based.

Multiple gestation was associated with a large increased risk of hemifacial microsomia in the patients studied by Werler et al {Werler, 2004 180 /id}, with a multivariate odds ratio of 10.5 (95% CI 4.2-26.2).

1.5.5 Bleeding in Pregnancy

Vaginal bleeding in pregnancy has been reported to be associated with adverse outcomes including pre term delivery, low birth weight, and increased perinatal death rates. Gestational vaginal bleeding has also been associated with an increased risk of congenital malformations in the resultant offspring. Sipilä et al {Sipila, 1992 91 /id} reported a prospective study of 8718 singleton pregnancies in Finland, and found that congenital malformations were significantly more common in women who had suffered from bleeding during pregnancy, odds ratio 2.9 (95 per cent CI 1.7-4.7). Strobino and Pantel-Silverman {Strobino, 1989 92 /id} prospectively studied 3,531 women in New York. They calculated an odds ratio of 1.7 (95 per cent CI 1.0-2.9) for the association of

first trimester bleeding and the presence of a congenital malformation in a live or still birth that was not due to a chromosomal anomaly; however, their confidence interval was wide and included parity. Batzofin et al {Batzofin, 1984 21 /id} found similar rates of congenital malformations in the offspring of 7229 mothers when a retrospective analysis of gestational bleeders versus non-bleeders was performed.

The only analysis reported in the literature of bleeding rates during pregnancy amongst mothers of hemifacial microsomia patients is that included in the study by Werler et al {Werler, 2004 181 /id}. First trimester bleeding was not associated with an increased risk of hemifacial microsomia. Second trimester vaginal bleeding was associated with an increased odds ratio of 13.2 (95% CI 2.3-75.8), but the numbers involved were small, 8 cases and 2 controls. Such an analysis involving a larger group of patients would be of interest in view of the vascular theory of the aetiology of hemifacial microsomia proposed by Poswillo.

1.5.6 Other aetiological theories

Lam {Lam, 2000 183 /id} observed that the pattern of abnormalities seen in hemifacial microsomia is similar to that seen in occult spinal dysraphism, a condition that presents as skin pits, tags, lipomas, and bony abnormalities in the lumbar region. It has been suggested that occult spinal dysraphism arises because of a failure of ectodermal separation, possibly because of a defect of cell adhesion molecules such as N-CAM and L-CAM, and Lam theorised that the same mechanism could be true for hemifacial microsomia. The ectodermal otic pit invaginates into mesenchyme, and normally its edges fuse to create the otic vesicle. The otic vesicle then migrates away from the surface ectoderm to eventually form the membranous labyrinth. Lam proposed that there was faulty separation of the otic vesicle from the surface ectoderm in hemifacial microsomia, and that the surrounding tissues are tethered and growth restricted. However, no experimental evidence exists to either confirm or refute this suggestion.

1.6 Genetic aspects

1.6.1 Mendelian and non-Mendelian inheritance

The expression of some genetic characters is dependent on the genotype at a single locus. These characters are termed Mendelian, named after the work of Gregor Mendel in the 1860's. The inheritance of a Mendelian character is said to be dominant if the character is expressed in a heterozygous individual, and recessive if it is only present in homozygotes. Mendelian characters may be determined by loci on the autosomal chromosomes or on the sex chromosomes, X and Y; X-linked characters may be dominant or recessive. It is often possible to distinguish which mode of inheritance is operating for a Mendelian character in studies of animals or plants. The advantages of these models over studies in humans include greater family sizes, shorter periods between generations, and the ability to perform test crosses in experimental animals or plants. However, when studying human families it is often difficult to identify the mode of inheritance of a character with any certainty. This uncertainty is further exacerbated by difficulties of data collection from individuals who made be widely separated in both time and place, and the possibilities of ascertainment bias, reduced penetrance, variable expression, imprinting, and the occurrence of new mutations.

Many characters in humans are not inherited in a simple Mendelian pattern. Obvious examples of this type of character include quantitative traits such as height, strength and intelligence. These characters, instead of being determined by the genotype at a single locus, may be governed either by a small number of loci, oligogenic, or a large number

of loci, polygenic, and they may also be influenced by environmental factors. The terms multifactorial or complex are used to describe these non-Mendelian characters. Multifactorial determination may also be involved in the inheritance of dichotomous or qualitative characters. The inheritance of a dichotomous character that is determined by multiple genes has been explained by the use of a polygenic threshold model; if the susceptibility to the character inferred by an individual's combination of genes is above a certain threshold then that character will be apparent, otherwise the character will be absent. Again, environmental influences may also play a role.

1.6.2 Mode of inheritance of hemifacial microsomia

Most cases of hemifacial microsomia are sporadic, with no evidence of Mendelian-like inheritance. However, some familial instances have been reported and examination of the pedigrees of these affected families most commonly suggests an autosomal dominant trait with variable expression {Hermann, 1969 108 /id} {Summit, 1969 107 /id} {Setzer, 1981 203 /id} {Regenbogen, 1982 52 /id} {Godel, 1982 159 /id} {Taysi, 1983 167 /id} {Robinow, 1986 166 /id} {Singer, 1994 53 /id}, {Stoll, 1998 54 /id}, {Kelberman, 2001 55 /id}. Overall, autosomal dominant inheritance is thought to occur in 1-2% of cases.

Rollnick and Kaye {Rollnick, 1983 57 /id} established pedigrees for 97 patients with microtia or hemifacial microsomia and found that 45% had relatives with ear anomalies,

mandibular hypoplasia, and/or early onset hearing loss. This seems to be a high proportion of cases with a positive family history, but examination of the data studied shows that the relatives classed as affected often had very mild ear malformations (see section 4.8). These findings may be interpreted as reflecting a multifactorial determination or autosomal dominant inheritance with variable penetrance. Kaye et al {Kaye, 1992 82 /id} performed segregation analysis on seventy four families with hemifacial microsomia and concluded that an autosomal dominant mode of inheritance was most likely; recessive and polygenic models of inheritance were not distinguishable, and the hypothesis of there being no genetic transmission was rejected.

Other pedigrees are more suggestive of an autosomal recessive mode of inheritance {Saraux, 1965 63 /id} {Krause, 1970 64 /id}. Brady et al reported the combination of hemifacial microsomia, external auditory canal atresia, deafness and Mullerian anomalies associated with acro-osteolysis, in a pedigree suggestive of autosomal recessive inheritance with variable penetrance {Brady, 2002 81 /id}.

Concordance and discordance have both been observed in monozygotic twins. Hemifacial microsomia has been reported in one of a pair of monozygotic twins in 14 cases {Burck, 1983 56 /id} {Stoll, 1984 60 /id} {Boles, 1987 161 /id}. Keusch et al {Keusch, 1991 204 /id} retrospectively identified 13 twin pairs wherein one individual exhibited hemifacial microsomia or hemifacial hypoplasia, but the zygosity was not identifiable from the data presented. There are three reports of hemifacial microsomia in one of a set of triplets following in vitro fertilisation {Yovich, 1985 13 /id} {Ferraris,

1999 205 /id} {Roesch, 2001 206 /id}. Concordance for the phenotype in monozygotic twins has been reported four times {Terhaar, 1972 153 /id} {Schweckendiek, 1976 65 /id} {Ryan, 1988 11 /id} {Keusch, 1991 204 /id}. Discordance of the signs of hemifacial microsomia in dizygotic twins has been found in 5 instances {Burck, 1983 56 /id} {Boles, 1987 161 /id}, whilst concordance for hemifacial microsomia is yet to be reported in dizygotic twins. These findings may be interpreted as supportive of a genetic cause for hemifacial microsomia, but could also indicate that environmental factors are more important (see section 4.10). Once the genetic loci involved in the development of hemifacial microsomia have been identified, and the disease genes are cloned, it should be possible to identify the inheritance pattern(s) for the deformity with some certainty, and it is likely that this will reveal a heterogeneous group of conditions with similar phenotypes.

1.6.3 Investigation of human disease genes

All the information required for cellular organisms to develop, function and reproduce is contained in cells in the form of deoxyribonucleic acid (DNA). The sequence of nitrogenous bases along the DNA molecule holds the information as a genetic code. The complete DNA sequence of an organism is termed its genome. Segments of the code, genes, can be deciphered by the intracellular apparatus to guide the synthesis of ribonucleic acid (RNA) which in turn specifies the production of polypeptides. Mutations of certain genes cause identifiable disease states, and much research effort is directed towards identifying these disease genes.

1.6.4 Chromosomal rearrangements

The starting point for identification of a disease gene may be a consideration of its chromosomal location. A candidate region for the location of a disease gene may be suggested by the occurrence of the disease in association with a chromosomal rearrangement that can be detected by cytogenetic analysis. A number of chromosomal rearrangements have been described in association with the hemifacial microsomia phenotype including pericentric inversion of chromosome 1 {Stahl-Mauge, 1982 160 /id}, del(5p) {Ladekarl, 1968 94 /id} {Neu, 1982 66 /id} {Choong, 2003 207 /id}, unbalanced translocation (5;8)(p15.31;p23.1){Josifova, 2004 172 /id}, del(6q) {Greenberg, 1987 141 /id}, trisomy 7 mosaic {Hodes, 1981 95 /id}, dup(7q) {Hoo,

1982 96 /id}, trisomy 9 mosaic {Wilson, 1983 42 /id}{de Ravel, 2001 162 /id}, pericentric inversion 9 {Stanojevic, 2000 170 /id}, tetrasomy 9p {Tonk, 1997 164 /id}, trisomy 18 {Bersu, 1977 97 /id}{Greenberg, 1987 141 /id}{Verloes, 1991 165 /id}, trisomy 18 mosaicism {Clarren, 1983 98 /id}, recombinant chromosome 18 {Sujansky, 1981 110 /id}, ring 21 chromosome {Greenberg, 1987 141 /id}, del(22q) {Greenberg, 1987 141 /id}{Herman, 1988 68 /id}, dup(22p) {Hathout, 1998 69 /id}, trisomy 22 {Kobrynski, 1993 70 /id}{Pridjian, 1995 171 /id}{de Ravel, 2001 162 /id}, 49,XXXXX {Schroeter, 1980 71 /id}, 49,XXXXY {Kushnick, 1975 72 /id}, and 47,XXY {Poonawalla, 1980 73 /id}. These reports may represent coincidental occurrences or actual relationships; a true association is more likely where multiple instances of the same link have been identified i.e. del(5p) and trisomy 18.

Potential loci for the disease gene may also be identified through mapping techniques, and these are discussed below. During the search for the gene that causes a particular disease, those genes that are most likely to be involved are selected for investigation and are termed candidate genes. Within a region of interest candidate genes can be selected according to their expression pattern or on the basis of their known functions.

1.6.5 Genetic mapping

The first attempts at identifying human disease genes involved strategies that did not require the localisation of a candidate chromosomal region as the first step. Genes were identified via knowledge of the protein product, which could be used to develop a gene-specific oligonucleotide probe, or through knowledge of a gene's DNA sequence or normal function. These position-independent strategies were adopted because sufficient mapping information was not available until the 1980s. Nowadays, the process of identifying a novel gene such as one involved in a genetic form of hemifacial microsomia usually begins with consideration of the chromosomal location of that gene.

Each copy of the human genome is organised into 23 chromosomes and most human cells are diploid, containing two copies of the human genome, one copy having been inherited from each parent. Thus, somatic cells contain two variants, alleles, of each gene. Diploid germ cells undergo a specialised form of cell division called meiosis to produce gamete cells that are haploid, containing only one copy of the human genome. During meiosis each pair of maternal and paternal homologous chromosomes become closely associated and may exchange segments; such crossover of DNA is termed recombination. These recombination events create the possibility of separation of any combination of genes on a single parental chromosome, and as a result any offspring will inherit a mixture of their parents' genes for each chromosome instead of an identical copy of one chromosome from one of their parents.

Mapping the location of a Mendelian disease gene is accomplished by the use of genetic markers. A genetic marker can be any Mendelian character which has a known chromosomal location. The aim in mapping is to discover how often the marker and the disease are separated by meiotic recombination; if the loci for the marker and the disease lie very close together on the same chromosome then recombination will rarely separate the two characters.

An individual possesses two alleles of the gene for a marker character and will pass one allele onto their child; in order to be able to determine which particular allele the child has inherited it is necessary for each parent to be differently heterozygous for that marker. Thus, a marker character must be polymorphic in order to be useful, and a marker with a greater number of alleles has a greater likelihood of being helpful in a mapping study. When it is possible to identify which variant of the marker is located on the same chromosome as the disease allele in an individual, the phase of linkage of the two characters is said to be known. If it is possible to determine whether or not a recombination has occurred between the marker and the disease locus in the course of a meiotic division, then that meiosis is said to be informative. Human genetic mapping requires the use of polymorphic genetic markers that are distributed throughout the genome. Currently, microsatellite markers are the standard tool for linkage analysis; these markers are repeats of a simple DNA sequence such as cytosine and adenine $(CA)_n$. Initial investigations into the usefulness of microsatellite markers concentrated on dinucleotide sequences because of their particular abundance; however, since microsatellite alleles may differ by only a single repeat the use of tri- and

tetranucleotide repeats has become increasingly popular since they are less prone to the production of multiple bands after polymerase chain reaction amplification and electrophoresis. Variations in the number of repeated blocks produce length polymorphisms. In the human genome there are 50 000 -100 000 interspersed $(CA)_n$ blocks {Weber, 1989 111 /id}. The polymerase chain reaction is used to amplify target DNA sequences containing the microsatellite markers, allowing a relatively small amount of an individual's DNA to be typed for markers that are chosen to be evenly spaced across the genome.

In recent years nucleotide variations at a single nucleotide site in the genome have also been used for genotyping. These single nucleotide polymorphisms are abundant throughout the genome, and whilst their mostly biallelic nature means that they are less informative than microsatellite markers it also renders them amenable to automated assessment.

1.6.6 Recombination fractions

For any two loci the proportion of meiotic divisions in which crossover of DNA between the loci occurs is known as the recombination fraction (θ) between the loci. The recombination fraction is a measure of the genetic distance between two loci located on the same chromosome; two loci that show recombination in 1 in 100 meioses (recombination fraction = 0.01) are defined as being 1centimorgan (cM) apart. Two

genes located on separate chromosomes (or far apart on the same chromosome) have a 50% probability of segregating together during meiosis and the recombination fraction between these loci is 0.5.

Recombination fractions do not exceed 0.5 however great the physical distance between the loci. The recombination fraction across a genetic map is not simply the sum of the recombination fractions between loci identified within that region. A mathematical conversion, the mapping function, is used to calculate the relationship between recombination fraction and genetic distance.

The physical distance, i.e. the number of nucleotides, represented by a unit of genetic distance is not constant across the genome, though the approximation of 1cM=1Mb averaged across the genome is often useful.

1.6.7 Linkage

If a disease gene and a marker are located on the same chromosome and their DNA sequences tend to be inherited together because of their physical proximity there is said to be linkage between the two loci.

Once a family is identified in which a Mendelian disease is segregating, and the individuals have been typed for an informative genetic marker, it is often impossible to

categorise every meiosis definitively as either recombinant or non-recombinant. However, if it is assumed that the loci are either linked (recombination fraction = θ) or not linked (recombination fraction = 0.5) a calculation can be made of the likelihood of the pedigree occurring. The ratio of the likelihoods at recombination fraction = θ and recombination fraction = 0.5 gives the odds of linkage, and the logarithm of this is the lod score. A positive lod score gives evidence in favour of linkage, a negative lod score gives evidence against linkage. Lod scores are usually calculated for a range of recombination fractions between 0 and 0.5, and the lod score will be highest at the most likely recombination fraction. A lod score of 3 is regarded as giving significant evidence of linkage, a lod score of –2 allows linkage to be rejected. These thresholds for significance are equivalent to 95% confidence limits. Computer programs are required for the calculation of lod scores for all but the very simplest of pedigrees because of the large number of calculations required.

Using data from multiple loci simultaneously may help to overcome problems due to some markers being limitedly informative. Typically a lod score is calculated for the unmapped disease locus at each marker position along a region of interest and a plot of marker location versus lod score can be generated; the highest peak indicates the most likely location of the disease locus.

Lod score calculations provide a powerful tool for performing linkage analyses, but they are not without their problems. Data errors and computational difficulties amongst other factors can lead to spurious results, and these difficulties are discussed further in section 6.2.

1.6.8 Genetic mapping in hemifacial microsomia in humans

Kelberman et al {Kelberman, 2001 55 /id} performed a genome wide search in a single family that displayed apparent autosomal dominant inheritance, and produced data that were highly suggestive of linkage to a region on chromosome 14q32.

Graham et al reported a large family that also exhibited likely autosomal dominant transmission of a Goldenhar-like syndrome{Graham, 1995 80 /id}. The affected members of the family had variable features including microtia, ear tags and ear pits, hearing loss, ocular and periocular dermoids, micrognathia, and seizures, but some individuals also had branchial cysts. The details of the study of this family have only been published in limited form as an abstract, and no mention is made of facial asymmetry. Linkage to 8q11-8q13 was identified in this family (maximum lod score 3.3 at $\theta = 0$), and this is the same region that harbours the gene that underlies branchio-oto-renal syndrome. The branchio-oto-renal syndrome is characterised by branchial arch

anomalies, e.g. pre-auricular pits, branchial fistulae or cysts, hearing loss, and renal dysplasia, and abnormalities of the pinna are also common {Jones, 1997 149 /id}. The syndrome is an autosomal dominant condition with variable expression, and the gene had been localised to chromosome 8q13 as noted above {Wang, 1994 195 /id} at the time of Graham et al's report. Since then the disease gene, *EYA1,* has been identified {Abdelhak, 1997 154 /id}. Hemifacial microsomia and branchio-oto-renal syndrome obviously share some clinical features, and a definitive clinical differentiation between the two conditions is not always possible in some individuals.

Chromosome 11q12-13 has also been proposed as a potential locus following analysis of an affected Australian family. The clinical details of this family have been published {Singer, 1994 53 /id} and they include nine affected individuals with varying features including microtia, pre-auricular skin tags, mandibular and soft tissue hypoplasia, macrostomia, and epibulbar dermoids. A genome wide scan produced a maximum multipoint lod score of 2.1 for the stated region on chromosome 11 {Kelberman, 2001 55 /id}, with the limited size of the family precluding the possibility of achieving a greater lod score.

1.6.9 Investigation of the genetic basis of complex diseases

As noted above, the inheritance of a genetically determined characteristic or disease may not follow a simple Mendelian pattern, but instead may be dependent upon the interaction of a number of separate genes and the environment. Segregation analysis may be used to estimate the most likely genetic contribution to a particular complex disease. Segregation analysis requires the collection of data from a large number of families affected by the non-Mendelian disease under investigation. The data is then analysed by a computer generated comparison of different modes of inheritance, gene frequencies, penetrances etc, to determine the mix of parameters which gives the highest overall likelihood for that data. Once the most likely genetic model has been defined, data from affected families can be subjected to parametric linkage analysis in a search for susceptibility loci. Segregation analysis has been performed for hemifacial microsomia as mentioned in section 1.6.2 and favoured autosomal dominant inheritance.

When mapping a complex disease in which there is no single major disease locus, traditional LOD score analysis of most affected families is unlikely to yield a significant result. However, sometimes families may be found that display a pattern consistent with near-Mendelian inheritance of the disease and standard parametric linkage analysis may be performed on data from these families. If disease gene loci are identified as a result of these analyses, they may relate only to a subset of the affected population that has a Mendelian condition which is phenotypically indistinguishable from the non-Mendelian

condition, or, because of chance occurrence of other determinants of the disease in the families studied, the identified loci may relate to susceptibility genes for the non-Mendelian condition. An example of this process is the identification of interferon regulatory factor 6 (*IRF6*) as a disease gene in isolated cleft lip and palate. *IRF6* was first discovered to be the cause of an autosomal dominant form of cleft lip and palate, Van de Woude syndrome, in which pits of the lower lip are the only additional remarkable feature {Kondo, 2002 194 /id}. A common single-nucleotide polymorphic variant was identified in this gene, and the same research group then performed transmission disequilibrium testing in 8003 individuals, which included 1968 families with a history of isolated cleft lip and palate. Strong evidence of over transmission of one allele of *IRF6* was found in families of probands with cleft lip with or without cleft palate, and the authors concluded that *IRF6* was responsible for 12 per cent of the genetic contribution to cleft lip and palate {Zucchero, 2004 193 /id}.

When a precise genetic model cannot be applied to an affected population or to a subset of the population, non-parametric linkage analyses e.g. affected sib pair analysis can be used to identify linkage to a chromosomal region. These analyses are based upon the non-random inheritance of a parental haplotype that is associated with the disease. Siblings who are affected by a genetic disease are likely to share a segment of the chromosome that carries the disease locus, and regions are therefore sought that are shared more frequently by the affected siblings than would be expected by chance.

Non-parametric genetic analysis can be applied to populations as well as to pedigrees, and these analyses are termed association studies. Association studies are based upon the assumption that alleles close to a disease locus tend to be co-inherited by affected individuals who have descended from a common ancestor. Thus, a search is made for linkage disequilibrium between known marker loci and the disease of interest in order to localise a disease susceptibility gene. The characterisation of an association is usually dependent on an appropriately chosen control group that allows calculation of the normal frequencies of the alleles and phenotypes under consideration. Association studies can also be conducted without a separate control population provided that the parents of the affected probands are available for typing. The transmission disequilibrium test is an example of this type of study. In this test probands and their parents are typed for the marker being studied. Parents who are heterozygous for the particular marker are selected, and the transmission disequilibrium test statistic examines the frequency with which the parents transmit the test allele to the probands compared to transmission of the other allele present in the parents. The practical application of this analysis has been mentioned above in the discussion of the genetic contribution of *IRF6* to isolated cleft lip or palate.

An example of a classically oligogenic disease is Hirschsprung disease (HSCR), in which there is congenital absence of ganglia in the large intestine. This disease provides a good model of the genetic analysis of a complex disorder. Segregation analyses initially suggested a dominant susceptibility to isolated HSCR in the long segment (L-HSCR) form of the disease, but other forms of the disease appeared to be either

multifactorial or due to a recessive gene with very low penetrance {Badner, Sieber, et al. 1990 91 /id}. Following the finding of an interstitial deletion of chromosome 10 in two patients, the disease susceptibility locus was mapped to 10q11.2 by pairwise and multipoint linkage analysis with microsatellite markers in 15 HSCR families{Lyonnet, Bolino, et al. 1993 92 /id}. Within this region the *RET* proto-oncogene was identified as a good candidate gene because it was known to be disease causing in multiple endocrine neoplasia type 2, which occurs concurrently with HSCR in some families. Mutations in *RET* were identified in HSCR patients, and subsequently mutations of the proto-oncogene *RET* have been identified in 50% of familial and 15-20% of sporadic cases of HSCR. Glial cell line derived neurotrophic factor (*GDNF*) was shown to be the *RET* ligand by phenotypic similarities between R*et*$^{-/-}$ and G*dnf*$^{-/-}$ knockout mice, and *GDNF* mutations have been found in a small number of HSCR patients, often in association with *RET* mutations or other contributory factors such as trisomy 21. Similarly a mutation of another *RET* ligand, neurturin (*NTN*) has been identified in one family in conjunction with a *RET* mutation. A separate susceptibility locus was mapped to 13q22 using genome-wide association studies {Carrasquillo, 2002 186 /id} {Puffenberger, 1994 187 /id}, and the positional candidate gene endothelin B receptor (*EDNRB*), that showed homology to a murine model of aganglionosis – the piebald-lethal gene, has since been implicated in HSCR. A mouse model for HSCR, dominant megacolon *(Dom)*, associated with Waardenburg syndrome allowed identification of the human homologue *SOX10* as another HSCR disease gene {Southard-Smith, 1998 188 /id}, though this is unlikely to be a major gene in isolated

HSCR. Presently eight genes have been shown to be involved in isolated Hirschprung's disease {Amiel & Lyonnet 2001 93 /id}.

As mentioned above, an important recent development in genetic mapping techniques has been the use of single nucleotide polymorphisms (SNPs) as genetic markers. These markers have only two alleles, but are more abundant than other commonly used genetic markers with an estimated average density in the human genome of 1 per 1000 base pairs {Wang, 1998 185 /id}. Genotyping using SNPs is amenable to automation because their bi-allelic nature allows simple plus/minus assaying, and it is possible to place thousands of oligonucleotide primers for different SNPs on a single solid state array. These DNA microarrays are called DNA or SNP chips, and whole genome analysis can be performed in a single hybridisation of the test DNA to such a SNP chip.

Hemifacial microsomia is most likely to be a complex disease; indeed, in view of the wide range of phenotypes that have thus far been diagnosed as hemifacial microsomia it is quite possible that a number of aetiologically different conditions are currently included in the discussions of this condition and its synonyms. The identification of families in which the condition is inherited in a Mendelian fashion suggests that a genetic influence is important in at least a proportion of cases, and added weight is given to this by the empirical recurrence risk of 1-2%. Evidence that more than one genetic locus is involved consists of the occurrence of features of the disease in association with a number of different chromosomal rearrangements, and from previous studies of dominantly affected pedigrees that have suggested linkage to three different

loci. However, it is also likely that environmental factors and the effects of non-disease genes have a major role in the pathogenesis of hemifacial microsomia as evinced by the great preponderance of sporadic over inherited cases, the modest concordance rate in monozygotic twins, and the increased incidence of affected children born to mothers with diabetes, or who have been exposed to various teratogens. The goal of this project was to use some of the methods of investigation described above to clarify our understanding of the genetic basis associated with hemifacial microsomia.

1.6.10 Animal models

As a result of close evolutionary relationships many different species have genes that possess significantly related sequences and functions, and these are termed homologous genes. Another approach for selecting candidate genes in the study of one species is to identify those genes in another species that are known to produce a similar phenotype, and then to study the structural and functional homologues of those genes. This method of investigation is commonly applied to the investigation of human disease genes via knowledge of the genetics of other animals which are more amenable to genetic studies. The mouse has been extensively genetically explored, and orthologous gene mutations are likely to produce similar phenotypes in the mouse and human. An example of the use of human-mouse phenotypic homology to identify a human disease gene is Waardenburg's syndrome type 1 (WS1). The Splotch (*Sp*) mouse mutant has pigmentary abnormalities, as has Waardenburg's syndrome in humans. Linkage analysis suggested localisation of the gene for WS1 to the distal part of human 2q, and the *Sp* gene mapped to mouse chromosome 1 that showed conservation of synteny with distal human 2q. The *Pax-3* gene was identified as a positional candidate gene mapping to the region of the *Sp* locus. The sequence of *Pax-3* was almost identical to an unmapped human genomic clone *HuP2*. These genes were shown to be orthologs and *HuP2* was re-named *PAX-3*.

Knockout and mutant animal models have been produced whose phenotypes include some of the craniofacial malformations characteristic of hemifacial microsomia. Otani

et al have reported a transgenic mouse line carrying an autosomal dominant insertional mutation that results in unilateral microtia and/or abnormal biting {Otani, Tanaka, et al. 1991 6 /id}. Histological examination of transgenic embryos displaying a second branchial arch malformation revealed haemorrhage in this region on the 9th day of gestation, which supports Poswillo's proposal of haemorrhage being the causal mechanism for hemifacial microsomia. The same research group {Naora, Kimura, et al. 1994 74 /id} mapped the site of integration of the transgene to chromosome 10 of the mouse and designated the mutational locus as "hemifacial microsomia-associated locus" or *Hfm*. The phenotype of this mouse model was extended by Cousley et al to include external auditory meatus hypoplasia, ossicular defects, tubotympanic hypoplasia, and temporomandibular joint underdevelopment {Cousley, Naora, et al. 2002 75 /id}. *Hfm* appears to be a strong animal model for hemifacial microsomia, but a disease gene within this region has not been identified as yet.

Juriloff et al reported that some mice that were heterozygous for the mutant gene first arch (*Far*) had hemifacial deficiencies as juveniles. Homozygotes for the *Far* mutation in another strain of mouse had bilaterally deficient maxillae, cleft palates, deficient lower eye lids and facial skin tags, but most heterozygotes with the same genetic background were normal {Juriloff, 1987 100 /id}. The *Far* gene has been mapped to chromosome 2 of the mouse {Juriloff, 1994 19 /id}.The general region in the mouse genome to which *Far* maps has homology with human chromosome 2q and 22 {Lyon, 1994 174 /id}, but the human homologue for the *Far* gene remains unidentified at present.

There are a number of mouse genes with known human homologues that have been shown to be involved in craniofacial development, and for which mouse mutants have been produced that display some of the characteristics of hemifacial microsomia. These genes are, therefore, potential candidate genes for hemifacial microsomia in man.

The genes that are involved in the regulation of hindbrain segmentation are important in the development of the ear and face. The mesenchymal tissue of each branchial arch consists of a core of cells derived from paraxial mesoderm surrounded by neural crest cells. The hindbrain is the source of the neural crest cells that populate the branchial arches {Fekete 1999 1 /id} and thus, abnormal hindbrain development can result in branchial arch anomalies reminiscent of hemifacial microsomia. The HOX genes are involved in the control of hindbrain segmentation and specification, and aplasia or hypoplasia of second arch derivatives, an absent or rudimentary auricle, and ossicular abnormalities were amongst the defects produced in *Hoxa1/Hoxb1* double mutant mice, but not in *Hoxa1* null mutants and *Hoxb1* null mutants {Gavalas, Studer, et al. 1998 3 /id}.

More candidate genes are to be found amongst the homeobox genes. These genes all contain a common DNA motif of about 180kb, and they have been shown to play important roles in early embryogenesis. Mice in which the homeobox gene, goosecoid (*Gsc*), has been knocked out display numerous craniofacial and rib cage abnormalities, including shortening of the mandible, underdevelopment of the muscular derivatives of the first pharyngeal arch, ossicular malformations, aplasia of the external auditory canal,

and a reduction in the size of the pinna {Rivera-Perez, 1995 101 /id} {Yamada, 1995 112 /id}. The *GSC* gene is located in the region 14q32, to which linkage has been suggested in a single, autosomal dominantly affected family with hemifacial microsomia; however, no mutations of the *GSC* gene were detected in the dominantly affected family or in 120 sporadic cases of hemifacial microsomia {Kelberman, 2001 55 /id}.

The *Dlx* homeobox gene family is also expressed within the embryonic craniofacial ectoderm. Qiu et al generated mice with mutations in *Dlx-1, Dlx-2*, and both *Dlx-1* and -*2*; all three mutants showed absence of the stapedial artery {Qiu, 1997 116 /id}. The three mutants also showed abnormalities of the derivatives of the maxillary division of the first arch, but the mandibular structures were normal.

The fibroblast growth factor 8 gene, *Fgf8*, has also been implicated as an epithelial signal that regulates gene expression during development of first branchial arch derivatives, and both *Dlx-1* and *Dlx-2* are targets for *Fgf8* {Shigetani, 2000 190 /id} {Thomas, 2000 133 /id}. Failure of development of the cartilaginous and skeletal structures derived from the first branchial arch was reported by Trumpp et al {Trumpp, 1999 119 /id} in mice in which *Fgf8* had been inactivated by cre-recombination. A proportion of mice heterozygous for this disruption was affected on only one side of the head and displayed a less severe phenotype.

Murine models have also suggested a possible role for abnormalities in endothelin regulation in the development of hemifacial microsomia. Endothelin-1 (*Edn-1*) is a signalling peptide expressed in the epithelial layer of the branchial arches. *Edn-1* $^{-/-}$ homozygous mice die at birth and have severe craniofacial malformations including poorly formed mandibles, hypoplastic auricles and absent auditory ossicles {Kurihara, 1994 113 /id}. *Edn-1* localises to human chromosome 6p24.

Deficiency of one type of endothelin receptor, *Ednra*, in mice mimics human velocardiofacial syndrome. The typical features of velocardiofacial syndrome include typical facies of a long face with vertical maxillary excess, malar flatness, prominent nose, and mandibular retrusion, narrow palpebral fissures, small auricles, cardiac anomalies especially ventricular septal defects, cleft palate and learning difficulties. Pharyngeal arch expression of *Gsc* is absent in *Ednra* receptor deficient mice suggesting that *Gsc* is a downstream signal triggered by activation of the *Ednra* receptor {Clouthier, 1998 115 /id}.

Edn-1 signalling is also relevant to another potential candidate gene, *Dhand. Dhand* is a transcription factor that is expressed in the mesenchyme underlying the branchial arch epithelium. *Dhand* homozygous null mice have hypoplastic branchial arches, though this appears to affect the third and fourth branchial arches more than the first and second arches. It has been found that *Dhand* is down regulated in the branchial arches of *Edn-1* null embryos {Thomas, 1998 114 /id}.

The retinoic acid receptor (Rar) family also provides candidates for hemifacial microsomia suggested by murine phenotypes. The family is comprised of three types of receptor, *Rar* α, β and γ, and their isoforms. Neural crest cell derived craniofacial skeletal elements were altered in double mutants of $\alpha\gamma$ {Lohnes, 1994 117 /id}. Many of the first, second and third pharyngeal arch-derived skeletal elements were malformed, though the mandible, temporomandibular joint and malleus appeared normal. A number of *Rar* double mutants also exhibited ocular abnormalities such as microphthalmia, retinal colobomas and corneal and conjunctival abnormalities.

Transcription factor *Ap-2*, also known as *Ap-2*α, is a retinoic-acid responsive gene. Homozygous disruption of the *Ap-2* gene results in complete failure of closure of the cranial neural tube; major alterations in the craniofacial skeleton, failure of closure of the ventral body wall, and forelimb anomalies are also seen. Chimeric mice composed of both wild-type and *Ap-2* null cells often had craniofacial anomalies independent of any neural tube closure defect {Nottoli, 1998 118 /id}. Malformations included cleft lip with or without cleft palate, mandibular and maxillary hypoplasia and dysmorphology. Ahituv et al {Ahituv, 2004 213 /id} used ENU-induced mutagenesis to produce a dominant mutation of *Ap-2*α named Doarad (*Dor*). *Dor* homozygous mice died perinatally with abnormal facial structures and ocular lens and retinal defects. *Dor* heterozygotes had ossicular malformations without any other observable phenotype.

Thus, there are a number of mouse gene mutations that produce phenotypes with deficiencies of first and second branchial arch derivatives similar to those described in hemifacial microsomia, and the human homologues of these genes are obvious candidates for the hemifacial microsomia disease gene in humans. The location of these genes should be borne in mind when examining the results of a linkage study for hemifacial microsomia.

Table 1. Summary of murine and human candidate genes for hemifacial microsomia

Candidate gene / locus	Murine chromosome
Far	2
Hfm	10, B1-3

Candidate gene	Human chromosomal region
DLX-2	2q31
DLX-1	2q31
RAR-β	3p24
EDNRA	4q32.2
DHAND	4q34
AP-2α	6p24
EDN-1	6p24
HOXA1	7p15.2
FGF8	10q24
RAR-γ1	12q13
GSC	14q32
RAR-α	17q21
HOXB1	17q21.3

1.7 Aims of Project

In order to investigate the genetic basis of hemifacial microsomia we set out to locate and identify the disease gene in a family ascertained by Dr R.J.Gorlin in which the features of the condition appeared to segregate in an autosomal dominant fashion. Linkage to disease gene loci suggested by other studies, i.e. 14q32, 8q11-13 and 11q12-13, was excluded in this family (M.Bitner-Glindzicz and J.Tyson personal communication). Therefore, we undertook a whole genome scan in order to identify the most likely locus for the disease gene, which would then allow a focused approach to the selection of candidate genes for further investigation.

We also decided to gather demographic, phenotypic and family information together with DNA samples from other patients with either hemifacial microsomia or isolated microtia, most of who would present as sporadic instances of these conditions, though we expected to identify some further familial cases. At Great Ormond Street Hospital a specialised multidisciplinary clinic has been established in order to optimise the management of patients with microtia. The clinic is attended by ENT surgeons, plastic surgeons, clinical geneticists, audiologists and prothetists, and receives referrals from across the United Kingdom. Since October 1998 a clinical research fellow has also attended the clinic with the prime aim of collecting data on the population of patients seen in the clinic.

The data from these patients would add valuable epidemiological data to that already within the public domain, and would allow comparison with similar data from other case series. In an attempt to gain a clearer understanding of the underlying mechanisms that cause hemifacial microsomia we collected data regarding those pregnancies that resulted in the birth of an individual with hemifacial microsomia, with particular attention to bleeding during pregnancy, and the occurrence of multiple gestations. The DNA samples were collected with a view to analysis for any specific genetic defect identified from the study of the large autosomal dominant pedigree.

2. Materials & Methods

2.1 Method of patient data collection

Children with microtia and/or hemifacial microsomia were identified from the group of patients referred to Great Ormond Street Hospital for assessment of these conditions in either the specialised microtia clinic, or in separate ENT, plastic surgery, clinical genetics, or maxillo-facial surgery clinics. Great Ormond Street Hospital in London is a paediatric tertiary referral centre and children are referred for treatment of microtia from all regions of the country. I attended all the bi-monthly microtia clinics and personally identified patients who were suitable for the study. The details of patients who were initially seen in the individual speciality clinics were sent to me by the consultants responsible for those clinics. I also attended the outpatient clinics of Mr David Gault at Mount Vernon hospital on a regular basis in order to identify other patients, both adults and children, with microtia and/or hemifacial microsomia. Mr Gault is a plastic surgeon with a particular interest in reconstructive surgery for microtia using autologous rib cartilage grafting, and patients with microtia are referred to him from around the country.

Ethical approval for the collection of patients' personal details, phenotype, past medical history and family history had been obtained from the Ethics Committees of Great Ormond Street Hospital and Mount Vernon Hospital by a previous research fellow attached to the ENT department of Great Ormond Street Hospital, and I updated these approvals as required during my period of data collection. I approached eligible patients and their families at their attendance in the outpatient department, or during an inpatient

admission, or by post, and obtained informed consent for their participation in this research project. The purpose and nature of the research project was explained verbally to the patients and their families, and each patient was given a printed information sheet (Appendix A). Patients and their accompanying family members were interviewed in order to obtain the following data: date of birth, sex, hospital identification number, contact details, general practitioner details, phenotype, past medical history including in utero and peri-partum details, and family history. Blood samples were collected from consenting probands and their available parents. Blood samples were also requested by post from parents who were not in attendance when the proband was seen at the hospital. DNA was extracted from all blood samples and stored by the North East Thames Regional Molecular Genetics Laboratory at Great Ormond Street for future DNA analysis. All of the collected data and details of the DNA samples obtained were recorded on a pro forma (Appendix B), and the data were then collated on a computerised database.

A subset of patients underwent reconstructive surgery for their microtia. During this type of surgery redundant skin in the region of the microtic remnant is often excised and discarded, and, again following informed consent, this tissue was collected together with a blood sample from the patient. Fibroblasts were extracted from the tissue samples and cultured in vitro. The resultant fibroblasts were stored in anticipation of a loss-of-heterozygosity study.

2.2 Materials

Taq polymerase, 10 x NH_4 reaction buffer, 50mM $MgCl_2$ and dNTPs were supplied by Bioline.

Electrophoresis grade agarose and 100bp ladder were supplied by GibcoBRL.

MegaBACE formamide loading solution, MegaBACE 10 x LPA buffer, MegaBACE long read matrix and ET-400R size standard were supplied by Amersham Biosciences.

All other chemicals were obtained from Sigma.

Primers

Research Genetics supplied microsatellite markers forming the publicly available Research Genetics Mapping Set 8.0. Polymerase chain reactions were performed at an annealing temperature of 51°C - 55°C with 1.5 mM – 3.0 mM $MgCL_2$. Primer sequences for additional markers were obtained from the Genome Database (www. gdb.org) as shown below, and these primers were synthesised by MWG Biotech. The annealing temperature for all the additional markers used was 55°C, with a magnesium concentration of 1.5mM.

Details of markers used that were not included in the Research Genetics Mapping Set 8.0

D2S117 Forward 5' GAGATCAGGTATATTCAATCCAC 3'

Reverse 5' CAGAAAATGACAAACTTTAGAGAG 3'

Expected product size 186-212 bp Heterozygosity 81%

Label used: Tet

D2S325 Forward 5' AGCTTCAGAAATGCCGTTG 3'

Reverse 5' CCAGTCTTCAGAGATAACAGGG 3'

Expected product size 190-214 bp Heterozygosity 79%

Label used: Hex

D2S371 Forward 5' AGAATAACTAACTTTGAGCAAGGTG 3'

Reverse 5' TCTGTATTGCATTATCTCTGTGTGT 3'

Expected product size 148-156 bp Heterozygosity 61%

Label used: Tet

D2S2361 Forward 5' CTGGACGACCAAGATTCTTCTAACC 3'

Reverse 5' CCTGGGCTCAAGCAATTCTC 3'

Expected product size 235–255 bp Heterozygosity 63%

Label used: Tet

D2S2382 Forward 5' ACATGCAACCGTGTGG 3'

Reverse 5' AGCACTCTTCAGTCCGAAC 3'

Expected product size 221–279 bp Heterozygosity 88%

Label used: Fam

D2S164 Forward 5' GTCCTAACAGGCCACAGACC 3'

Reverse 5' GCTGGCAGTATCACATGACA 3'

Expected product size 265–303 bp Heterozygosity 93%

Label used: Tet

D2S2179 Forward 5' TCCCCAGTGTCATTTCC 3'

Reverse 5' GCAGACTTGTTCAGAGAGC 3'

Expected product size 106–122 bp Heterozygosity 56%

Label used : Tet

D2S2151 Forward 5' CCTGCACTCTCATGTATATTG 3'

Reverse 5' GTGCCTGACTTATTTTACTTTG 3'

Expected product size 208–252 bp Heterozygosity 46%

Label used: Hex

D2S1242 Forward 5' TGACATAGCGAGACCCTGTC 3'

Reverse 5' CCATTCTCATCCAGCAGGA 3'

Expected product size 117–172 bp Heterozygosity 85%

Label used: Hex

D2S339 Forward 5' GAAGTCATGGGAAGGC 3'

Reverse 5' AAGCAAAATGCAATCAGA 3'

Expected product size 123–133bp Heterozygosity 89%

Label used: Fam

D2S2228	Forward 5' AGCTGTAAGGAAGCAGCAC 3'	
	Reverse 5' AAAAGGACTTATTCTGGGGA 3'	
	Expected product size 191–215 bp	Heterozygosity 71%
	Label used: Tet	

Solutions

5 x TBE	0.089M Tris, 0.089M Boric acid, 2mM EDTA, pH 8.0
Agarose gel loading dye	50% glycerol, 10mM EDTA, 0.5% Orange G

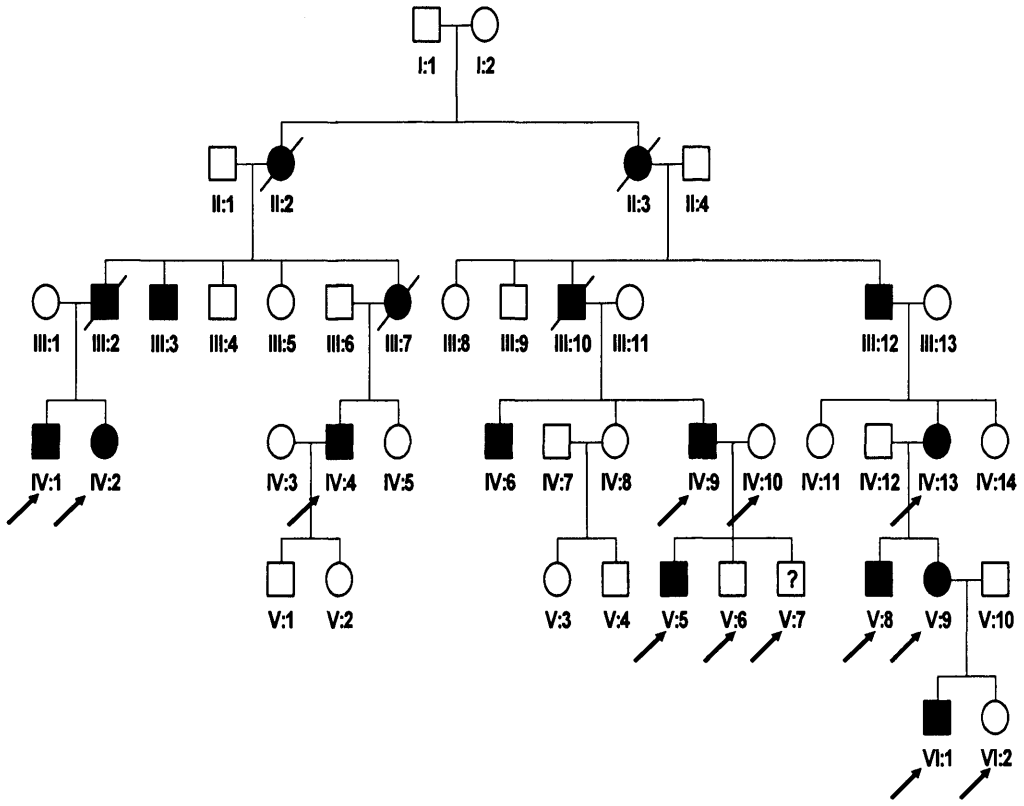
2.3 Methods of genome scan

Details of a family from North America, in which successive generations showed characteristics of hemifacial microsomia, were supplied by Dr Robert Gorlin, University of Minnesota, USA. DNA samples from a number of family members were also provided by Dr Gorlin.

2.3.1 Clinical details of patients studied in genome scan

Family R is the North American family identified by Dr R.J. Gorlin. Some details were provided about 6 generations of the family in which hemifacial microsomia appears to segregate in an autosomal dominant manner with apparently full penetrance (figure 5). Clinical details of the family were also supplied by Dr Robert Gorlin; individual IV_4 has left-sided mandibular hypoplasia, with masseter underdevelopment, preauricular skin tags, an unstable temporomandibular joint, and a small right-sided preauricular fistula. Individuals IV_{13}, V_8 and V_9 were all said to be affected, and all exhibited preauricular skin tags. The first information received stated that individual V_7 was unaffected. Further details were then supplied that included the fact that V_7 had skin tags, and we chose to include him as an affected individual at the start of the genome scan. However, later examination of individual V_7 by Dr M. Bitner-Glindzicz revealed that he exhibited no other features of hemifacial microsomia, and, thus, his affection status was called into question during the course of this study.

Figure 5. Pedigree of family R exhibiting autosomal dominant hemifacial microsomia. Arrows indicate individuals for whom DNA samples were initially available.



A genome scan was performed using the above details and the available DNA from family R. The Research Genetics Mapping Set version 8.0 that was used contains 496 markers covering the autosomal chromosomes. These markers are spaced at an average interval of 10cM across the genome. A series of polymerase chain reactions was performed on each individual's DNA with each marker in turn. The products of the

polymerase chain reactions were analysed using a combination of gel electrophoresis and automated analysis in the MegaBACE 1000 instrument.

2.3.2 Polymerase chain reaction

The polymerase chain reaction mix consisted of 25-50ng of DNA in a 10µl final volume containing 10-20pmol of each primer, 1 x NH_4 reaction buffer (Bioline), 1.5-3.0mM $MgCl_2$, 200µM dGTP, dCTP, dTTP, dATP, and 0.1-0.2 units of Taq polymerase (Bioline). Each sample was overlaid with a single drop of mineral oil to reduce evaporation. PCR reactions were performed in 96-well Omniplates (Hybaid), or 0.5ml eppendorf tubes.

The reaction was carried out on a Hybaid Omn-E thermal cycler.

Conditions for thermal cycling consisted of denaturation at 95°C for 5 minutes, followed by 35 cycles of 95°C for 30 seconds, 30 seconds at the annealing temperature specific to each primer pair which ranged from 51 °C to 55 °C, and 72°C for 30 seconds, followed by a final extension step of 10 minutes at 72°C.

2.3.3 Gel electrophoresis

1g of electrophoresis grade agarose was dissolved in 50ml 1 x TBE by boiling in a microwave to produce a 2% agarose gel. On cooling 3µl of 10mg/ml ethidium bromide solution was added to the mix and the gel was poured into an NBL gel tray. 10µl of PCR product were mixed with 3µl of loading dye, loaded onto the gel and electrophoresed at 100 volts for 25-45 minutes in 1 x TBE. The gel image was captured using ChemiImager 4000 software (α Innotech) under ultraviolet (UV) light. Products were sized by comparison to a 100bp ladder loaded at the same time as the PCR products.

2.3.4 Capillary array electrophoresis

The MegaBACE 1000 instrument was used to perform automated capillary gel electrophoresis. The MegaBACE capillary tips were rinsed with deionised filtered water according to the manufacturer's protocol, and then the capillaries were filled with MegaBACE long read matrix. The PCR products to be run in the same capillary were pooled in equal ratios and diluted to between 1 in 10 and 1 in 30 in order to desalt the pools. 4.0µl of diluted PCR pooled products were added to 5.5µl of formamide loading solution and 0.5µl ET-Rox 400 size standard ladder. The prepared samples were loaded onto Thermo-Fast 96 well skirted PCR plates (ABgene) and denatured at 95°C for 1 minute and placed immediately on ice prior to injection into the capillaries and

electrophoresis according to the MegaBACE protocol. Electrophoresis was performed for 75 minutes at 44°C running at 750 volts.

2.3.5 Data analysis

The data acquired from the PCRs and electrophoreses were analysed using the Genetic Profiler v1.1 analysis software (Amersham). The electropherograms generated by this software were individually assessed in order to call and size alleles appropriately. Two point lod score analyses of the family's genotypes for each microsatellite marker were performed using the MLINK program from the LINKAGE software package (www.hgmp.mrc.ac.uk). The number of alleles of each marker present in the general population and their frequencies were obtained from the database of the Laboratory of Population Genetics at the National Cancer Institute (http://lpg.nci.nih.gov) and CEPH genotype database (www.cephb.fr/cephb/). The disease penetrance was set at 100%, and the hemifacial microsomia disease gene frequency was set at 1 in 5000. Parametric multipoint lod score analysis was performed with GENEHUNTER version 2.0 (www.hgmp.mrc.ac.uk), using Kosambi mapping units, and an increment distance of 1cM.

3. Results of patient data collection

Over a four year period until October 2002 two hundred and sixty one patients with either isolated microtia or hemifacial microsomia gave their informed consent to be interviewed and examined, as described in the methods section above, and for their data to be retained on the database; I personally recruited 107 of these patients to the database. Ninety five patients displayed clinical features consistent with a diagnosis of hemifacial microsomia, and one hundred and sixty six patients appeared to have isolated microtia.

3.1 Age and sex distribution

The year of birth of the patients recruited to the database is shown in figures 6 and 7. The number of patients recruited from each year of birth is modest, as expected considering the total number of patients in the study. Most of the patients were recruited at Great Ormond Street Hospital which is a tertiary referral centre for paediatric patients. Mount Vernon hospital accepts both adult and paediatric patients, and 29 patients, 20 children and 9 adults, of the 166 patients with microtia were recruited from Mount Vernon Hospital, as were 13 of the 95 patients with hemifacial microsomia of whom 8 were children at the time of presentation. Therefore, 95% of our patients presented in childhood, and one might intuitively expect this pattern to occur, though the likelihood of an early presentation may have been accentuated in our study by the nature of the institutions from which we recruited patients. However, some patients do present in adulthood, the oldest patient in each group being in their mid forties at their first assessment in clinic.

Figure 6. Year of birth distribution of microtia patients

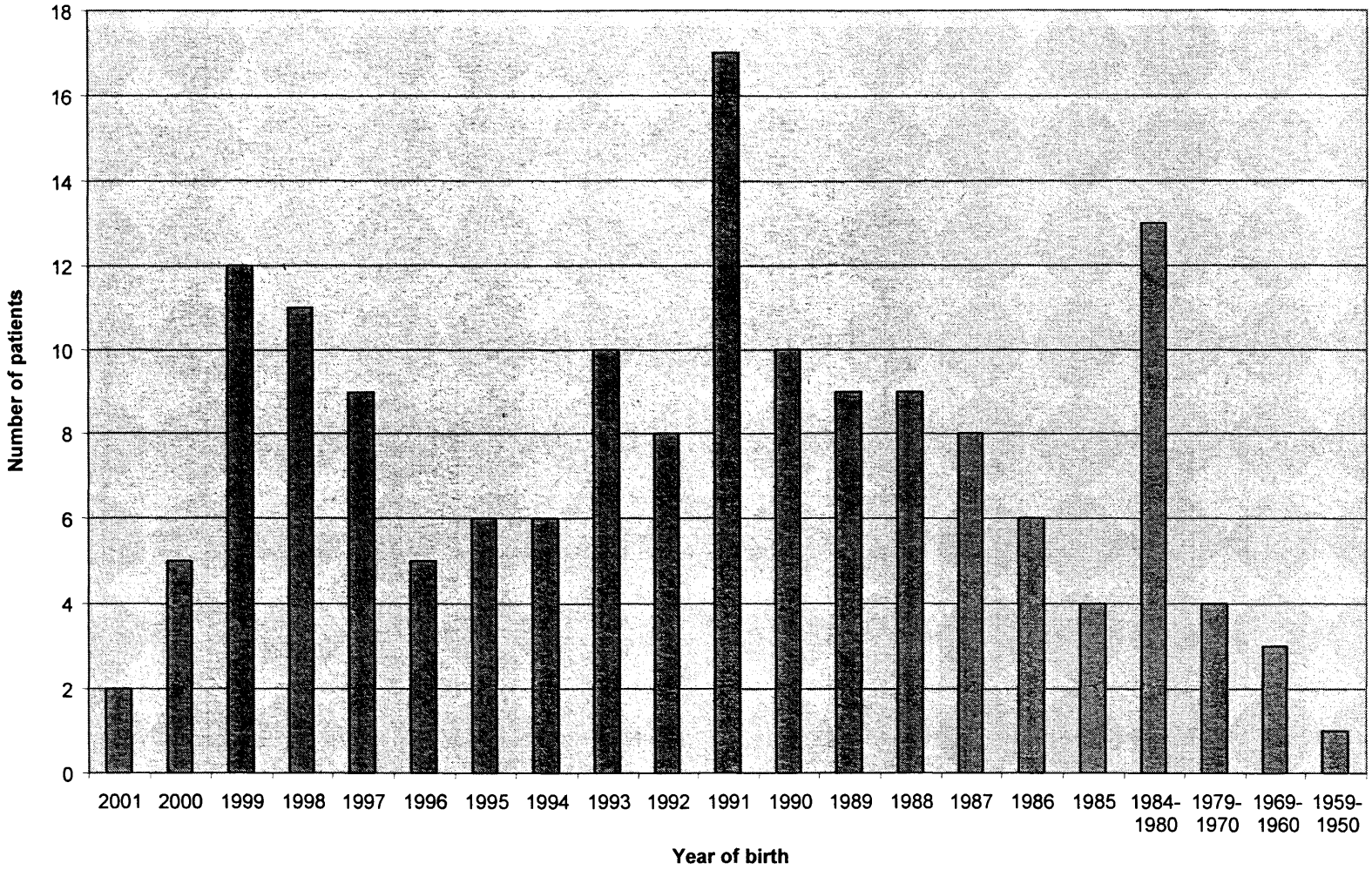
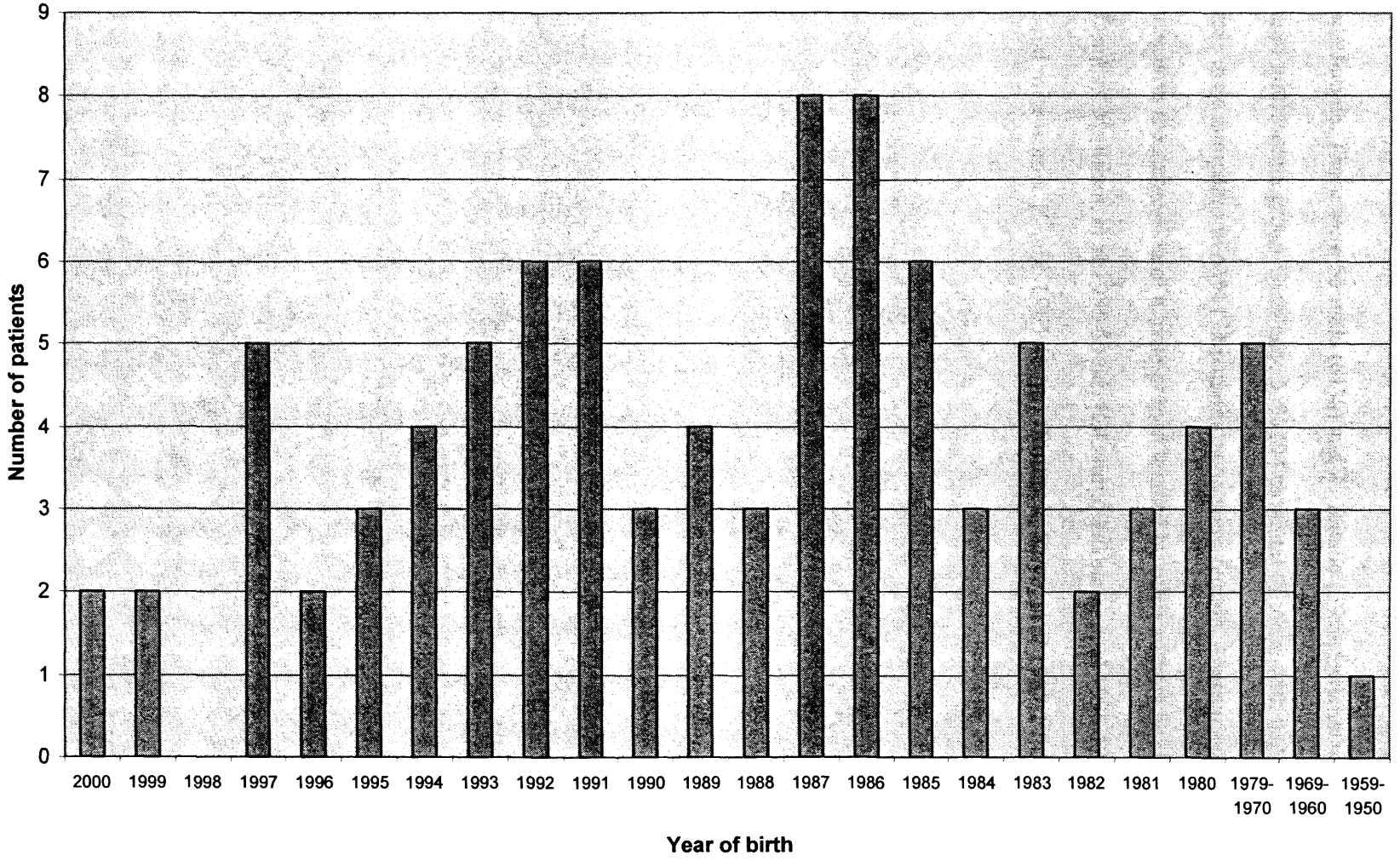


Figure 7. Year of birth distribution of hemifacial microsomia patients



Male patients were more common than females, with an overall ratio in the 261 patients of 1.4:1 male:female. There were 58 male (61%) and 37 female (39%) patients within the hemifacial microsomia group. Similarly, 57% (94 patients) of those with isolated microtia were male compared to 43% (72 patients) who were female. These proportions are consistent with the suggestion of a male preponderance by other authors in both microtia {Bennun, Mulliken, et al 1985 50 /id} {Okajima, Takeichi, et al 1996 123 /id} and hemifacial microsomia {Grabb 1965 46 /id} {Rollnick, Kaye, et al 1987 2 /id}.

3.2 Laterality

A predominance of right sided facial asymmetry has been reported in most published series of patients with hemifacial microsomia {Grabb 1965 46 /id} {Rollnick, Kaye, et al 1987 2 /id} or microtia {Bennun, Mulliken, et al 1985 50 /id} {Okajima, Takeichi, et al 1996 123 /id}.

Right sided laterality was more common in our patients with isolated microtia, with 96 out of 166 patients (58%) displaying an isolated right microtia, 42 patients (25%) showing unilateral left microtia, and 28 patients (17%) presenting with bilateral microtia.

There was also a right sided preponderance in the hemifacial microsomia patients in terms of both facial hypoplasia and microtia. In 53 patients (56%) of the 95 with hemifacial microsomia the right side of the face was hypoplastic, in 37 patients (39%) only the left side of the face was affected, and in 3 patients (3%) there was bilateral facial microsomia; in two patients the side of facial hypoplasia was unrecorded. 47 (49%) of the 95 hemifacial microsomia patients had unilateral right sided microtia, 32 patients (34%) had unilateral left microtia and 9 patients (9%) had bilateral microtia. The microtic ear was always on the same side as the facial hypoplasia in those patients with unilateral hemifacial microsomia. 7 patients had facial asymmetry in the absence of microtia.

3.3 Grade of microtia

We have graded the severity of microtia in our patients using a modification of the grading system proposed by Meurman {Meurman 1957 102 /id}. In this system grade I microtia is defined as a small pinna in which all parts of the normal ear can be identified; grade II is a pinna in which some but not all parts of a normal ear are observed, and in grade III microtia the pinna is represented by ill defined mass of tissue or anotia is present. Other grading systems have also been described, but none has any clear diagnostic or prognostic advantage, and the Meurman system has the advantage of simplicity. The grade of microtia was recorded for 83 of the patients with hemifacial microsomia, 9 of whom had bilateral microtia, and in 159 patients with isolated microtia, 26 of who had bilateral microtia. The number of ears assigned to each grade of microtia is shown below.

Table 2. Laterality and grades of microtia observed

Grade of microtia	Hemifacial microsomia patients		Microtia patients	
	Right ear	Left ear	Right ear	Left ear
Grade 1	15	9	30	19
Grade 2	16	9	67	29
Grade 3	20	23	21	19

3.4 Skin tags

Pre-auricular skin tags were a common finding in both groups of patients, but the incidence of skin tags was higher in the hemifacial microsomia patients than in the isolated microtia patients, 48 (51%) of the 95 patients with hemifacial microsomia had skin tags compared to 36 (22%) of the 166 patients with isolated microtia. The numbers of skin tags and their sides of presentation are shown in table 3.

Table 3. Number and laterality of pre-auricular skin tags.
(percentages are of the total number of patients in either the hemifacial microsomia or isolated microtia groups)

Number of skin tags	Hemifacial microsomia patients		Microtia patients	
	Right	Left	Right	Left
1	15 (16%)	16 (17%)	22 (13%)	14 (8%)
2	13 (14%)	6 (6%)	4 (2%)	3 (2%)
3	2 (2%)	3 (3%)	0	0
4	1 (1%)	1 (1%)	0	0

Unilateral skin tags were most commonly seen to be ipsilateral to a microtic ear, though in some patients the skin tag was located on the opposite side to the microtic ear. These contralateral skin tags were found in 9 of the 29 patients who had isolated microtia and unilateral skin tags. For the purpose of this study these patients were considered to have unilateral microtia. Out of the 38 hemifacial microsomia patients who had unilateral skin tags and microtia, ipsilateral skin tags were seen in 26 patients and contralateral skin tags occurred in 12 patients. One hemifacial microsomia patient had two unilateral skin tags contralateral to the side of facial hypoplasia in the absence of any microtia.

Bilateral skin tags were reported in 9 patients with hemifacial microsomia, 2 of whom had bilateral facial hypoplasia and bilateral microtia, whilst another patient had unilateral facial hypoplasia with bilateral microtia; bilateral tags were seen in 7 patients in the microtia group, 3 of who also had bilateral microtia.

3.5 Abnormalities of the external auditory meatus

Stenosis and atresia of the external auditory meatus were common findings in both groups of patients. The majority of patients had unilateral narrowing or occlusion of their ear canals as shown in table 4.

Table 4. The incidence of unilateral and bilateral narrowing of the auditory meatus (percentages are of the total number of patients in either the hemifacial microsomia or isolated microtia groups)

External auditory meatus stenosis or atresia	Hemifacial microsomia	Microtia
Bilaterally normal	17 (18%)	37 (22%)
Unilaterally affected	70 (74%)	112 (67%)
Bilaterally affected	8 (8%)	17 (10%)

The number of normal, stenotic and atretic ear canals found in our populations is shown in table 5 below. The incidence of atresia of the external auditory meatus is greater than that of canal stenosis, though the later character was only subjectively assessed.

Table 5. Distribution of variations in patency of external auditory meati (percentages are of the total number of ears in either the hemifacial microsomia or isolated microtia groups)

External auditory meatus	Hemifacial microsomia	Microtia
Normal	104 (55%)	171 (52%)
Stenosis	28 (15%)	20 (6%)
Atresia	58 (30%)	141 (42%)

Tables 6 and 7 below detail the number of patients with either ear canal stenosis or atresia for whom the grade of associated microtia had been recorded. These figures appear to suggest that meatal atresia is more common in association with the more severe degrees of microtia in both groups of patients.

Table 6. Relationship of ear canal narrowing to grade of microtia in hemifacial microsomia patients. (percentages are of the total number of ears for each grade of microtia)

Hemifacial microsomia patients Microtia grade	External auditory canal	
	Stenosis	Atresia
Normal	1 (1%)	0
I	12 (50%)	8 (33%)
II	7 (28%)	14 (56%)
III	6 (14%)	35 (81%)

Table 7. Relationship of ear canal narrowing to grade of microtia in isolated microtia patients. (percentages are of the total number of ears for each grade of microtia)

Microtia patients Microtia grade	External auditory canal Stenosis	 Atresia
Normal	0	0
I	10 (20%)	19 (39%)
II	6 (6%)	82 (85%)
III	4 (10%)	33 (83%)

No instances were recorded of canal atresia in relation to a normal pinna. Only one patient from either group was noted to have auditory canal stenosis ipsilateral to a normal pinna, and the contralateral ear was microtic. I have classed this patient as having unilateral microtia.

3.6 Facial nerve palsy

A number of patients were noted to have a seventh cranial nerve palsy. This was seen more commonly in the hemifacial microsomia group of patients, of whom 42% had a facial palsy, whilst only 3% of the 166 patients with isolated microtia were similarly affected. The facial palsy was always ipsilateral to the side of microsomia or microtia.

A right sided facial palsy was seen in 23 patients (24%) of the 95 with hemifacial microsomia, and in 2 patients (1%) from the isolated microtia group. A left facial palsy occurred in 17 (18%) hemifacial microsomia patients and in 3 (2%) microtia patients.

3.7 Cleft lip and palate

Five patients (3%) of the isolated microtia group had some form of clefting of the lip or palate. One had a bilateral cleft lip and palate, one patient had a submucous cleft palate, two patients had midline palatal clefts, and one had a bifid uvula.

Eleven per cent of the hemifacial microsomia group had a cleft lip and/or palate; there were four patients with midline cleft palates, two patients had bilateral cleft lips, there was one right sided palatal cleft, and three left sided clefts.

3.8 Affected relatives

The majority of patients appeared to be sporadic occurrences of hemifacial microsomia or microtia. However, a number of probands had isolated relatives who also had microtia or another malformation that could be a feature of hemifacial microsomia, and these are summarised in tables 9 and 10. There were two cases in which a significant family history of multiple affected relatives was elicited.

The pedigree of one proband with hemifacial microsomia included facial asymmetry affecting his mother, maternal grandmother and mother's first cousin, and pre-auricular skin tags were present in a maternal cousin (figure 8).

Microtia was reported in the father, great grandfather and great great grandfather of a separate patient who had bilateral grade III microtia, and his great uncle had pre-auricular skin tags. This family has been reported in the literature as familial microtia suggestive of autosomal dominant inheritance with variable expression and low penetrance {Gupta & Patton 1995 208 /id}. Unfortunately there was no opportunity to confirm the findings in these two pedigrees by direct examination of the affected individuals.

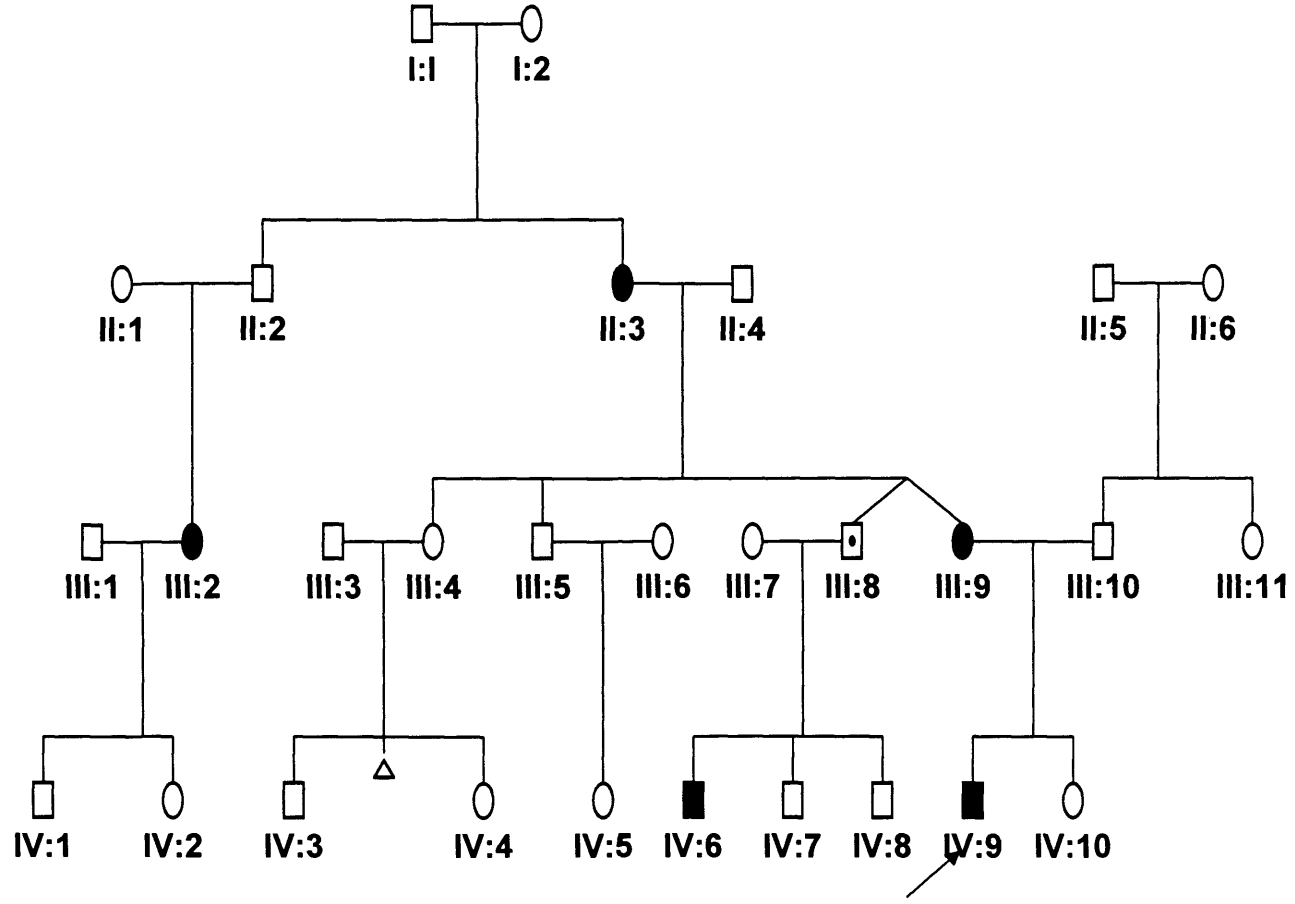


Figure 8. The pedigree for the family with hemifacial microsomia affecting three generations. Facial asymmetry was present in IV:9, III:9, II:3 and III:2, and pre-auricular skin tags were present in IV:6.

The incidence of all relatives bearing ear deformities or facial asymmetry is shown in table 8 below.

Table 8. Relatives with ear deformities or facial asymmetry

Affected relatives	Hemifacial microsomia	Microtia
First degree	5 (5%)	2 (1%)
Second degree	4 (4%)	6 (4%)
Third degree	3 (3%)	5 (3%)

Table 9. Congenital anomalies present in relatives of hemifacial microsomia probands

	Proband database ID number
Sibling with cupped ear	398516
Maternal grandfather with cupped ear	398516
Maternal grandmother with facial asymmetry	646495
Sibling with clinodactyly	646495
Mother's cousin with cleft palate	566242
Mother's uncle with cleft palate	566242
Sibling with microtia	B0203068
Sibling with unilateral hearing loss	665190
Mother with facial asymmetry	598360
Mother with cleft lip and palate	597036
Father with pre-auricular skin tags	535057
Father with microtia, mandibular hypoplasia and iris coloboma	150897
Father with unilateral microphthalmia	661139
Maternal grandfather with microtia	589824
Maternal great grandfather with microtia	B0216064
Mother's cousin with microtia	100565
Sibling with Down's syndrome, family history of hearing loss	665227
Paternal uncle unilateral hearing loss from childhood	736939

Table 10. Congenital anomalies present in relatives of probands with isolated microtia

	Proband database ID number
Sibling with pre-auricular skin tags n=4	629918, B0177003 703417, B0179123
Mother with cupped ears	717088
Mother with pre-auricular skin tags	723907
Father with pre-auricular skin tags	730766
Father with pre-auricular pit	723929
Paternal grandfather with bilateral microtia	710285
Paternal grandmother with pre-auricular skin tags	705196
Maternal grandmother with microtia	B0173804
Cousin with pre-auricular skin tags	733483
Paternal cousin with lop ear	617231
Maternal cousin 'malformed ears'	B0196611
Maternal cousin once removed with microtia, mandibular hypoplasia and facial palsy	B0196611
Cousin with microphthalmia	646714
Maternal aunt with microtia n=2	B0213781, 740005
Maternal uncle with microtia	715733
Paternal aunt with microtia	669645
Maternal aunt with syndactyly	662352
Sibling with solitary kidney	650167

3.9 Bleeding in pregnancy

Bleeding during pregnancy was reported more frequently by mothers of patients with hemifacial microsomia than isolated microtia. In the hemifacial microsomia cohort 27 of the 95 mothers (28%, 95%CI 19%-37%) reported bleeding in their first trimester of pregnancy, and 3 (3%, 95%CI 0%-7%) recalled bleeding in the second trimester. The numbers of mothers of microtia patients reporting bleeding were 14 (8.4%, 95%CI 4.2%-12.7%), 4 (2.4%, 95%CI 0%-4.7%) and 2 (1.2%, 95% CI 0%-2.9%) in each trimester of pregnancy respectively.

Mothers were also questioned about bleeding during other pregnancies for comparison. 70 mothers of children with hemifacial microsomia gave a history of 121 other gestations, and all of these mothers had at least one other live born child. 5 of these 70 mothers reported bleeding during one of their other pregnancies, which equates to bleeding in 4% of these mothers' other gestations. Only one mother reported bleeding during the gestation of their child with hemifacial microsomia and also during another gestation. 2 mothers of individuals with hemifacial microsomia reported spontaneous first trimester miscarriages.

119 mothers of children with microtia reported 225 other gestations, of which 213 ended with a live birth. 13 (6%) of these 213 pregnancies were complicated by bleeding, though one mother accounted for four of these pregnancies. 6 of the pregnancies with bleeding occurred in mothers who also noted bleeding during their carriage of the child

with microtia, including the woman mentioned above whose five gestations were all associated with spotting in the first trimester; 7 pregnancies were complicated by bleeding in 6 women who had not noticed bleeding during their pregnancy that gave rise to the child with microtia.

3.10 Incidence of twinning

Six (6%, 95% CI 0%-13%) of the hemifacial microsomia patients and eight (5%, 95% CI 2%-8%) of the microtia patients were born as one of a pair of live born twins. All of the twin siblings of affected individuals were phenotypically normal. In each group one mother of an affected individual was said to have had two foetuses in utero early in their pregnancy, but by the second trimester only a singleton foetus remained, and another patient was delivered with two sets of amniotic membranes.

4. Discussion of patient data collection

Hemifacial microsomia is a complex disorder that presents with a highly variable phenotype consisting particularly of facial and auricular abnormalities. It is quite probable that the spectrum of abnormalities that has been encompassed by the epithet of hemifacial microsomia actually includes a number of aetiologically distinct disorders. Whether patients with isolated microtia represent the mildest form of hemifacial microsomia, or whether they constitute a separate diagnostic condition is unclear at present, and this distinction will only be made with certainty once the aetiology and pathogenesis of both defects have been defined. We have collected data on a large number of patients of whom roughly a third have clinical features consistent with hemifacial microsomia, whilst two thirds have microtia without overt hypoplasia of the mandible or maxilla. The presence or absence of other malformations in association with microtia was determined by history taking and clinical examination alone. We did not perform cephalometric radiography or photography routinely, and it is possible that minor degrees of facial asymmetry could have been overlooked, especially in small infants. Also it has been noted that facial asymmetry may only become apparent in some patients following the differential growth of the normal and affected sides of the face in the first few years of life; on-going planned review of our patient set in the dedicated microtia clinic at Great Ormond Street Hospital and continued updating of the database should allow any such individuals to be correctly reclassified.

4.1 Age and Sex distribution

The demographic distribution of our patients reflects the referral pattern to the microtia clinic at Great Ormond Street Hospital at which most of the patients were recruited. Hence, most of the patients were under 16 years of age at presentation. In keeping with the non progressive nature of microtia and hemifacial microsomia, and the absence of a need for early assessment of affected patients, many patients were first referred in mid childhood, and we would expect more patients who were born in the late 1990's and 2000/2001 to present to the clinic in the coming years.

A male preponderance was present in our study group, the ratio of males to females being 1.4 to 1, with 152 males and 109 females in total, and this difference appears to be significant (95% CI's males 52%-64%, females 36%-48%). This ratio was similar for both the patients with isolated microtia and for those with hemifacial microsomia. These findings are consistent with most other reported series. Rollnick et al {Rollnick, 1987 2 /id} calculated a male to female ratio of 1.9 to 1 (male:female 191:103) in a series of patients with either isolated microtia or hemifacial microsomia, and this relationship was maintained when the microtia subgroup (male 64%, female 36%) and hemifacial microsomia subgroup (male 65%, female 35%) were inspected separately. The same male to female proportions, 65% to 35%, were found in 592 patients with microtia studied in Japan {Okajima, Takeichi, et al 1996 123 /id}, and in 74 microtia patients (male 66%, female 34%) in Massachusetts, USA {Bennun, Mulliken, et al 1985 50 /id}. Eavey {Eavey 1995 124 /id} reported a less marked male preponderance (male:female

60%:40%) in 92 paediatric patients most of whom had microtia, though there were 21 ears out of a total of 108 with other auricular malformations such as cryptotia. The same 1.5:1 male:female ratio was reported by Llano-Rivas et al {Llano-Rivas, Gonzalez-del Angel, et al 1999 184 /id} amongst 145 patients in Mexico City with either microtia or hemifacial microsomia.

However, Carvalho et al {Carvalho, Song, et al 1999 51 /id} found no bias towards male sex (male 46%, female 54%) in their group of 99 paediatric patients with hemifacial microsomia, and another group from Massachusetts found a similar result in a smaller number of patients (male 43%, female 57%, n=40) {Rahbar, Robson, et al 2001 86 /id}. An earlier study of 121 patients by the same group showed equal numbers of affected boys and girls (male 49%, female 51%) {Horgan, Padwa, et al 1995 125 /id}. The series of 154 patients with hemifacial microsomia and/or microtia reviewed by Vento et al {Vento, LaBrie, et al 1991 209 /id} in America contained 85 (55%) female and 69 (45%) male patients. A more detailed analysis of the data in each of these four reports suggests that there is no difference in their proportions of male and female patients.

4.2 Laterality

The predominantly right sided laterality of our patients' malformations, of both isolated microtia and hemifacial microsomia, is in keeping with the findings of other case series. Unilateral microtia was seen in 138 (83%) of the 166 patients in the isolated microtia group, and 96 (70%, 95%CI 62%-77%) of these 138 patients had a right sided deformity. Rollnick et al {Rollnick, Kaye, et al 1987 2 /id} reported on 92 patients with microtia alone, in 42% of whom only the right ear was affected, in 18% the left ear was affected, and in 36% there was bilateral involvement. Harris et al {Harris, Kallen, et al 1996 175 /id} found an excess of right sided cases amongst 615 unilateral cases of microtia, with 61% being right sided. Okajima et al {Okajima, Takeichi, et al 1996 123 /id} reported a large series of 592 patients with microtia. Despite the fact that a small percentage of these patients had malformations consistent with known syndromes e.g. Treacher-Collins syndrome in 1%, a similar pattern in the sides affected by microtia was seen; the right side was affected in 58.4%, the left side in 32.4%, and 9.1% were bilaterally affected. Bennun et al's {Bennun, Mulliken, et al 1985 50 /id} series of 74 patients showed right laterality in 57%, left laterality in 30% and bilateral microtia in 13%; these figures are similar to ours of 58%, 25% and 17% respectively. Amongst 87 patients with microtia Llano-Rivas {Llano-Rivas, Gonzalez del-Angel, et al 1999 184 /id} noted that 46% had right unilateral microtia, 23% left microtia and 31% had bilateral affection.

Horgan et al {Horgan, Padwa, et al 1995 125 /id}, however, found that side predominance was distributed more equally in their series of 121 patients between right, left and bilateral cases (34% right, 38% left, 28% bilateral).

In our group of patients who had hemifacial microsomia together with microtia the pattern of involvement was again repeated, right, left and bilateral microtia occurring in 53%, 36% and 10% of patients. The pattern of microtia laterality in our hemifacial microsomia patients is similar to that found by Llano-Rivas {Llano-Rivas, Gonzalez del-Angel, et al 1999 184 /id}, whose 145 patients comprised 52% right sided, 23% left sided and 25% bilateral. Rollnick et al {Rollnick, Kaye, et al 1987 2 /id} also reported 199 patients with microtia and mandibular hypoplasia; they found a 31% bilateral microtia rate (62 patients), with 41% (81patients) and 28% (56 patients) of cases having unilateral right or left sided involvement only.

With regards to facial asymmetry the right side of the face was more commonly the side of hypoplasia than the left, this being the case in 57% compared to 40%. We noted bilateral facial involvement in only 3 patients (3%), and whilst this may represent the true proportion of bilaterally affected subjects, it must be borne in mind that this was assessed subjectively without the use of measurement and comparison to anthropometric normalised data. Grabb quoted a figure of 12% for bilateral facial involvement in hemifacial microsomia, 12 of his 102 patients being affected on assessment by clinical examination and, in many cases, x-ray analysis {Grabb 1965 46 /id}. Bilateral mandibular hypoplasia was evident in 16% of the series reported by

Vento et al {Vento, LaBrie, et al 1991 209 /id}, with little difference in the proportion of patients with either unilateral right (45%) or left (40%) hypoplasia.

Paulozzi and Lary {Paulozzi & Lary 1999 178 /id} examined laterality patterns in 6,390 infants with external birth defects. A statistically significant excess of right sided cases was noted for six defect types, and these included microtia and preauricular sinuses. A significant left sided excess was present for nine defect types, one of which was preauricular tags.

The theories that have been expounded to account for asymmetric occurrence of external birth defects include the constraining effect of the uterus once the foetal head is engaged, since the foetus tends to face to the mother's right side. However, this is unlikely to be an important factor in the development of the structural defects seen in hemifacial microsomia or microtia as the foetal head does not engage in the pelvis until the 28^{th} week of gestation. An increased susceptibility of the right side of the embryo to hypoxic tissue damage has been suggested by Fantel et al {Fantel, Juchau, et al 1989 196}. The same group has also shown that mitochondrial activity develops on the left side of the embryo before the right side, and this could lead to lower energy reserves and increased tissue damage from hypoxia on the right side {Fantel, Person, et al 1991 198 /id). Subtle differences in the vascular patterns of the two sides of the embryo could also lead to an excess of hypoxic damage on one side of the body.

The establishment of a left-right axis occurs early on in embryonic development, and studies have shown that some genes are differentially expressed on the two sides of the embryo {Ramsdell & Yost 1998 179 /id}. Such differences in gene expression could potentially cause one side to be more susceptible to other adverse genetic or environmental influences, and thus explain the generally consistent finding of the preponderance of right sided involvement in hemifacial microsomia and microtia.

4.3 Grade of Microtia

The assignment of certain appearances of microtic ears to distinct categories is somewhat arbitrary, as noted in the introduction. Most grading systems define the mildest form of microtia as a structurally normal ear that is small in size, and this is usually determined by comparison to the contralateral ear. More severe grades of microtia are defined by the increasing loss of normal parts of the ear, with the severest form being complete loss of any tissue representing an ear, i.e. anotia. The very mildest forms of microtia might go unrecognised, especially if there is bilateral involvement. A small but normally shaped ear may not be of great concern to an individual or their parents, or may not be deemed worthy of referral to a tertiary centre by the local medical services, and thus our population may be skewed towards the more severe end of the spectrum of microtia deformities. Only 30 (18%) of the 166 patients with isolated microtia had a unilateral grade one deformity of the pinna, and 6 (4%) had bilateral grade one microtia.

Few other authors have reported the distribution of severity of microtia in their case series. Rollnick and Kaye's population of hemifacial microsomia patients had a fairly even distribution between the different grades of microtia; of the 47 microtic ears 17 (36%) were grade I, 19 (40%) were grade II, and 11 (23%) were grade III {Rollnick & Kaye 1983 57 /id}. Okajima et al divided their 592 patients with microtia into 5 groups according to the classification of Ogino published in Japanese. They reported that grade III microtia, peanut-shell type, was the most frequent type having been observed in 56.8%. Grade II microtia was seen in 20.6%, and grade I microtia in 13.9% {Okajima, Takeichi, et al 1996 123}.

4.4 Skin Tags

Pre-auricular skin tags were a more common finding in the hemifacial microsomia group of patients than in the isolated microtia group. 49% (n=47) of the 95 people in the hemifacial microsomia cohort had associated skin tags, compared to 22% (n=36) of the 166 people with isolated microtia. The incidence of skin tags in both groups of our study appears to be much higher than that observed in the general population, an incidence of 1.5% being quoted by Altmann {Altmann 1951 173 /id}, but it remains unclear as to whether skin tags have an aetiology which is directly related to the cause of hemifacial microsomia.

21 of the 24 Canadian patients with Goldenhar syndrome studied by Shokeir had preauricular skin tags {Shokeir 1977 152 /id}. 40% of the 154 hemifacial microsomia patients reported on by Vento et al {Vento, LaBrie, et al 1991 209 /id} had preauricular skin tags. A much lower percentage of patients was found to have preauricular sinuses or tags in the study by Llano-Rivas {Llano-Rivas, Gonzalez del-Angel, et al 1999 184}, either in association with microtia alone (17.2%), or in their patients with hemifacial microsomia (18.9%).

We found the presence of skin tags contralateral to the side of microtia in 12 (15%) of 79 patients with hemifacial microsomia and unilateral microtia, and in 9 (7%) of 138 patients with unilateral isolated microtia, and until there is a greater understanding of the development of the microtic defect it is debatable as to whether such patients should be categorised as being unilaterally or bilaterally affected.

4.5 Abnormalities of the External Auditory Meatus

Amongst the patients with hemifacial microsomia the external ear canal was stenotic in 15% of ears (28/190) and atretic in 31% (58/190). The comparative figures for the isolated microtia group were 6% (20/332) and 42% (141/332) respectively. These proportions are statistically significantly different (χ^2 $0.01 > p > 0.001$), with most of the difference being attributable to the increased rate of stenosis observed in the hemifacial microsomia patients. Higher rates of atretic ear canals were found by Llano-Rivas

{Llano-Rivas, Gonzalez del-Angel, et al 1999 184 /id} in their patients, with 57% of their microtia patients and 52% of their hemifacial microsomia patients displaying this defect.

We made the assessment of the presence of stenosis of the ear canal subjectively, and minor degrees of stenosis may have been missed. However, minor stenosis of the ear canal is likely to be clinically unimportant. Atresia of the external auditory meatus is clinically more likely to be associated with a significant conductive hearing loss, and presents a greater barrier to the treatment of any associated hearing impairment.

4.6 Facial nerve palsy

Facial nerve palsy was found to occur much more commonly in association with hemifacial microsomia, 42% (95% CI, 32%-52%), than with isolated microtia, 3% (95% CI, 0%-6%). The aetiology of these facial nerve palsies is assumed to be an aberration of the facial nerve at some point along its intraosseous course within the temporal bone. The temporal bone does not originate from the first or second branchial arches, apart from the styloid process, but it is not hard to conceive that the mechanism(s) causing disruption of the normal development of the structures derived from these branchial arches could sequentially affect the contiguous portions of the temporal bone. Rahbar et al {Rahbar, Robson, et al 2001 86 /id} examined temporal bone computed tomographic (CT) scans of 40 patients with hemifacial microsomia, and found abnormalities of the facial nerve canal in 35 (88%) of these. Anterior

displacement of the facial nerve was the most commonly noted abnormality, and was found in 25 patients (63%). Clinically, 20 of these patients (50%) had a facial nerve weakness.

Facial nerve dysfunction was found in 22 (22%) of 99 paediatric patients with hemifacial microsomia in a retrospective case note review performed by Carvalho et al {Carvalho, Song, et al 1999 51 /id}. The same incidence of facial nerve palsy was reported by Bassila and Goldberg {Bassila & Goldberg 1989 126 /id} in their published series of 50 patients with hemifacial microsomia.

Bennun et al {Bennun, Mulliken, et al 1985 50 /id} in their report of 74 patients with microtia found facial nerve weakness in 9 patients (12%), and Eavey {Eavey 1995 124 /id} reported an 8% facial nerve palsy rate in his series of 92 paediatric patients with auricular malformations. Okajima et al {Okajima, Takeichi, et al 1996 123 /id} reported a much lower incidence of facial nerve palsy, 13 (2%) of their 592 patients with microtia having an ipsilateral facial weakness.

Thus, it appears that facial palsy is more commonly associated with hemifacial microsomia than with isolated microtia; this difference could be attributable either to different pathogeneses of the two conditions, with the pathogenesis of hemifacial microsomia being more likely to produce a facial nerve palsy, or because hemifacial microsomia represents a more severe phenotypic expression of the same condition as microtia, with the anatomical abnormalities being more likely to extend to the temporal bone and the course of the facial nerve.

4.7 Cleft Lip and Palate

Clefting of the lip and/or palate is the commonest congenital malformation of the head and neck with an incidence of 1 in 1000 {Gorlin, Cohen, et al 1990, 211 /id} {ICBDMS 2002 61 /id}. Fourteen patients in our total study group of 261 were found to have clefting of the lip and/or palate, which represents an incidence of 5%. Llano-Rivas et al {Llano-Rivas, Gonzalez del-Angel, et al 1999 184 /id} noted 4 cases (3%) of cleft palate amongst their 145 patients with microtia. Rollnick et al {Rollnick, Kaye, et al 1987 2 /id} reported the incidence of cleft lip +/- cleft palate as 7% in association with either microtia or hemifacial microsomia. Feingold and Baum {Feingold & Baum 1978 16 /id} had 3 patients with cleft palate in their series of 16 Goldenhar patients, and Shokeir {Shokeir 1977 152 /id} reported cleft lip and palate in 25% of their 24 Goldenhar patients.

Thus, cleft lip and or palate are seen to occur in a small percentage of the larger series of microtia or hemifacial microsomia, though the incidence appears to be greater than in the general population. The incidence may seem surprisingly low if one considers that the lip and palate are formed from the tissues of the maxillary processes of the first branchial arch, and that normal fusion in the midline of these tissues occurs at the same time as the development of the pinna.

4.8 Affected Relatives

The incidence of affected relatives was much less amongst our patients than has previously been reported in another series {Rollnick & Kaye 1983 57 /id} {Llano-Rivas, Gonzalez del-Angel, et al 1999 184 /id}. In our patients a family history of microtia or facial asymmetry was identified in 16 pedigrees in total (6%), with 8 of the probands having hemifacial microsomia and 8 having isolated microtia. If relatives with milder ear malformations e.g. cup or lop ears, preauricular skin tags, pits or sinuses, and early onset hearing loss are considered affected, as in the series reported by Rollnick and Kaye, then 31 pedigrees (12%) could be classified as showing a positive family history. Llano-Rivas et al {Llano-Rivas, Gonzalez del-Angel, et al 1999 184 /id} elicited a family history of abnormalities in 33% of their 145 patients with microtia with or without hemifacial microsomia. Rollnick and Kaye presented pedigree data on 97 propositi, 44 (45%) of whom had a family history of affected relatives. Although the proportion of patients with an affected relative appears much greater in Rollnick and Kaye's series than in our own, the difference may be mitigated by their recording of more extensive family histories. A comparison of the frequencies of affected first, second and third degree relatives reveals a much greater similarity between the two groups. In total the frequencies of affected first, second and third degree relatives in our series were 6.1%, 4.6% and 1.9% respectively, and these figures are similar to the 8%, 3% and 1% reported by Rollnick and Kaye. A more detailed examination of the data presented by Rollnick and Kaye also shows that their criteria for classifying a relative as affected are quite broad. They considered a relative to be affected if he or she had

hemifacial microsomia, isolated microtia, and/or unilateral mandibular hypoplasia, but they also included relatives who were reported to have preauricular skin tags or pits, cleft lip and/or palate, "crumpled" helix, "thin" pinna, large "pointed" ears, and early on-set hearing loss before the age of 20.

Okajima et al {Okajima, Takeichi, et al 1996 123 /id} recorded data on 592 patients with microtia, including six patients with Treacher-Collins syndrome; 15 of these patients (2.5%) had relatives with ear malformations. This percentage is lower than that in our series of patients, in which 7 of the 95 patients with hemifacial microsomia (7%) had relatives with ear malformations, and 10 of the 166 patients with microtia (6%) had a family history of ear abnormalities. Aase {Aase, 1980 127 /id} reported 11 familial cases (9.5%) amongst 116 individuals with microtia in New Mexico. A review of 92 paediatric patients with auricular malformations, amongst whom there were 72 microtic ears {Eavey 1995 124 /id}, yielded a family history of external ear anomalies in 9 cases (10%).

4.9 Bleeding in pregnancy

The rate of gestational bleeding in our total population of 261 patients was 19% (50 patients, 95% CI 14% to 24%) which is not significantly different to rates reported in studies of general maternity populations. Strobino and Pantel-Silverman {Strobino & Pantel-Silverman 1989 92 /id} noted that vaginal bleeding occurred at sometime during pregnancy in 22% of 3,531 women, and a survey of 17000 British births in 1970 reported a similar incidence (21.7%). A higher rate of gestational bleeding might have been expected in our study; Ornoy et al {Ornoy, Benady, et al 1976 210 /id} reported rates of bleeding during pregnancy of 33% and 29%, in patients with either congenital abnormalities of the central nervous system, or congenital abnormalities of other systems respectively.

The overall incidence of bleeding at anytime during pregnancy reported by mothers of patients with hemifacial microsomia was 32% (95% CI 22%-41%) and most of these patients' mothers recalled bleeding during the first trimester of their affected pregnancies, (28%, 95% CI 19% to 37%), which contrasts with generally reported rates of bleeding during the first trimester that range between 7% {Sipila, Hartikainen-Sorri, et al 1992 91 /id} and 12% {Williams, Mittendorf, et al 1991 128 /id}. A prospective survey of 550 pregnant, normal women in Hampshire recorded a 21% incidence of bleeding in the first 20 weeks of pregnancy, though 67 (12%) of these pregnancies ended in miscarriage; thus, the incidence of bleeding in those pregnancies who had a live birth would have been 9% (95% CI 6.7% to 11.5%){Everett 1997 129 /id}. The

possibility of an increased rate of early gestational bleeding in association with hemifacial microsomia is of interest in view of the vascular and hemorrhagic aetiological theories that have been mentioned above.

4.10 Incidence of Twinning

The rate of twinning in England and Wales is 1.4% {Macfarlane, Mugford, et al 2000 147 /id}. The rates of twinning in our populations were 6.3% (n=6, 95% CI 1.4% to 11.2%) and 4.8% (n=8, 95% CI 1.6% to 8.1%) in the hemifacial microsomia group and microtia group respectively. Only the proband was affected in each pair of twins identified in our study. Literature reports of discordance and concordance amongst twin pairs have been discussed in section 1.6.2. Overall, the concordance rate for monozygotic twins for hemifacial microsomia is nearly 20%. For most malformations or malformation complexes the concordance in monozygotic twins is usually in the range of 10%-20% {Schinzel, Smith, et al 1979 89 /id}. The higher concordance rate found in monozygotic twins compared to dizygotic twins that has been recorded in hemifacial microsomia can be taken as evidence of a genetic contribution. However, the concordance rate for monozygotic twins for hemifacial microsomia is well below 50%, and may mean that environmental factors have a larger influence in the development of the phenotype. Similarly, Schinzel {Schinzel, Smith, et al 1979 89 /id} has suggested that the occurrence of discordance in monozygotic twins indicates that the disorder is

the result of a single early localised malformation rather than a genetically predetermined syndrome.

5. Results of genome scan

5.1 Analysis of candidate loci

Previous work has tried to exclude potential candidate loci as the region containing the disease gene in family R (M. Bitner-Glindzicz and J. Tyson personal communication). Candidate loci were selected on the basis of the reported linkage of two separate families with hemifacial microsomia to chromosomes 8q11-8q13 {Graham, Hixon, et al. 1995 80 /id} and 14q32 {Kelberman, Tyson, et al. 2001 55 /id} respectively. In addition a putative locus on chromosome 11q12-11q13 had been identified in an Australian family with hemifacial microsomia with a maximum multipoint lod score of 2.1 {Singer, Haan, et al 1994 53 /id} (personal communication J. Goldblatt). Individuals from whom DNA was available were genotyped for markers flanking and within the above loci. The two point lod scores obtained were not suggestive of linkage and multipoint lod scores for all three regions were less than -2 (personal communication M. Bitner-Glindzicz and J. Tyson). Thus, linkage to all three regions was excluded in family R.

Simulation analysis of three fully informative markers in this family, with penetrance assumed to be 100% and with a disease gene frequency of 0.0002, suggested a maximum multipoint lod score in excess of 4 was achievable. Therefore, a genome wide search for linkage was performed to identify the disease locus.

5.2 Genome search

Individuals from family R for whom DNA was available were genotyped using the microsatellite markers comprising the Research Genetics screening set version 8.0, with an average density across the entire genome of approximately 10cM.

5.2.1 Two point linkage analysis

For each marker with which family R was genotyped, two point lod scores were calculated using the MLINK program from the LINKAGE software package (www.hgmp.mrc.ac.uk). Initially the analysis was performed using data from all the individuals (both affecteds and unaffecteds) who had been successfully genotyped. In order to overcome potential problems arising from the possibility of reduced penetrance the analysis was then repeated with the unaffected individuals IV:10, V:6 and VI:2 classed as genotype unknown, so that only affected individuals were included in the analysis. The two sets of two point lod scores produced by these two analyses are shown in tables 12 to 33.

The initial pedigree data we obtained indicated that individual V:7 was not affected for hemifacial microsomia. At the start of the project further details received from Dr Robert Gorlin mentioned that individual V:7 had preauricular skin tags and it was

therefore assumed that he was affected. The MLINK lod scores were calculated on this basis, and individual V:7's genotyping data was included in the affecteds only analysis. However, subsequent reassessment of this individual's phenotype has cast some doubt as to his affection status. He had been born with a single pre-auricular skin tag that was removed in childhood, and displayed no sign of facial asymmetry or any other feature of hemifacial microsomia (M. Bitner-Glindzicz personal communication). In order to determine what difference a change in the affection status of individual V:7 would make on the results of the lod score analysis, the GENEHUNTER version 2 software (www.hgmp.mrc.ac.uk) was used to recalculate two point lod scores for the same set of markers that were analysed with MLINK, but with the affection of individual V:7 classed as unknown. The results of this analysis are also included in tables 12 to 33. Due to the memory constraints of the software used, just the affecteds only analysis was repeated with the GENEHUNTER program. The GENEHUNTER program only produces a single value for each marker at a recombination fraction of zero.

Table 11 summarises the results of the genome scan, highlighting the markers for which the highest two point lod scores were achieved. Tables 12 to 33 contain all of the results from the three sets of two point lod scores: the MLINK analysis of affecteds and unaffecteds (with V:7 classed as affected), the GENEHUNTER analysis of affecteds only (with V:7 classed as affection unknown), and the MLINK analysis of affecteds only (with V:7 classed as affected).

Table 11. Summary of notable two point lod score results.

(recombination fraction (θ)=0 unless otherwise stated)

Marker name	Chromosomal band identifier	Maximum Two Point Lod Score MLINK analyses, either all individuals or affecteds only (V:7 affected)	Maximum Two Point Lod Score GENEHUNTER analysis, affecteds only (V:7 unknown)
D5S1471	5q34 –5q35	3.18	3.18
D3S4531	3q25	2.34 (θ=0.1)	2.86
D5S1470	5p14	2.17	1.87
D11S2359	11q25	1.86	1.87
D13S895	13q34	2.82	1.83
D2S1370	2q32 – 2q37	1.56	1.67
D8S1985	8q24	1.02 (θ=0.1)	1.68
D16S749	16p12	1.34	1.34
D3S2403	3p25	1.89 (θ=0.1)	-2.39
D8S1459	8q22	1.51	0.92
D10S1427	10q22	1.51 (θ=0.1)	-2.38

Table 12. Chromosome 1 two point lod score results

Marker name	Distance to next marker (cM)	MLINK analysis, affecteds and unaffecteds, V:7 classed as affected						GENEHUNTER analysis, affecteds only, V:7 classed as unknown		MLINK analysis, affecteds only, V:7 classed as affected						Multipoint graph locus number
		Recombination fraction								Recombination fraction						
		0	0.1	0.2	0.3	0.4				0	0.1	0.2	0.3	0.4		
D1S2132	4.4	-infinity	-0.01	0.16	0.13	0.07		-infinity		-infinity	0.43	0.35	0.19	0.06		1
D1S1612	7.2	-infinity	-2.11	-0.97	-0.42	-0.13		-infinity		-infinity	-1.35	-0.52	-0.16	-0.02		2
ATA9B08	7.6	-infinity	-1.98	-0.86	-0.36	-0.12		-infinity		-infinity	-1.36	-0.54	-0.21	-0.06		3
D1S1597	7	-infinity	-0.13	0	0.23	0.12		-infinity		-infinity	-0.87	-0.34	-0.14	-0.05		4
D2S1368	8.8	0.91	0.64	0.39	0.2	0.07		0.91		0.91	0.64	0.39	0.2	0.07		5
D1S552	7.1	-infinity	-0.9	-0.39	-0.17	-0.06		-3.46		-3.46	-0.31	-0.13	-0.06	-0.02		6
D1S1676	4.9	-6.27	-0.49	-0.15	-0.05	0.02		-6.57		-6.57	-0.54	-0.18	-0.06	-0.02		7
D1S1622	5.6	-5	-0.17	0.13	0.17	0.11		-6.40		-6.13	-0.33	-0.03	0.03	0.03		8
GATA137F01	11.9	-infinity	-0.59	0	0.13	0.09		-infinity		-infinity	-0.92	-0.26	-0.04	0.01		9
GATA129H04	3.4	-infinity	0.79	0.77	0.53	0.27		-infinity		-infinity	0.06	0.23	0.2	0.11		10
D1S2134	10.3	-infinity	-1.34	-0.5	-0.16	-0.02		-infinity		-infinity	-1.62	-0.74	-0.34	-0.12		11
D1S1150	8	-infinity	-1.59	-0.56	-0.18	-0.05		-infinity		-infinity	-2.21	-0.104	-0.49	-0.19		12
GATA165C03	9.4	-1.27	1.04	0.66	0.21	-0.08		-2.17		-1.27	1.04	0.66	0.21	-0.08		13
D1S1613	5.4	-infinity	-1.05	-0.34	-0.11	-0.05		-infinity		-infinity	-1.75	-0.83	-0.4	-0.15		14
D1S1630	6.1	-2.38	-0.18	0.02	0.01	-0.03		-2.68		-2.68	-0.34	-0.05	0	-0.01		15
D1S1665	8.7	-2.32	0.37	0.31	0.16	0.04		-2.56		-2.56	0.07	0.08	0.04	0		16
D1S1728	2.9	-infinity	-1.13	-0.43	-0.17	-0.06		-infinity		-infinity	-1.46	-0.64	-0.27	-0.09		17
D1S551	5.1	-infinity	0.63	0.54	0.34	0.15		-infinity		-infinity	0.12	0.13	0.06	0.01		18

Marker name	Distance to next marker (cM)	MLINK analysis, affecteds and unaffecteds, V:7 classed as affected						GENEHUNTER analysis, affecteds only, V:7 classed as unknown		MLINK analysis, affecteds only, V:7 classed as affected						Multipoint graph locus number
		Recombination fraction								Recombination fraction						
		0	0.1	0.2	0.3	0.4				0	0.1	0.2	0.3	0.4		
D1S2129	3.5	-infinity	-0.23	0.05	0.07	0.04		-2.09		-1.79	0.7	0.5	0.25	0.07		19
D1S1588	10.8	-infinity	-0.95	-0.42	-0.17	-0.05		-infinity		-3.51	-0.27	-0.02	0.03	0.02		20
GATA45B07	7.4	-7.39	-1.1	-0.48	-0.19	-0.05		-6.67		-6.67	-0.7	-0.27	-0.09	-0.02		21
D1S1631	2.8															
GATA176G01	7.2	-infinity	-0.62	0.2	0.15	0.09		-infinity		-infinity	-0.62	0.02	0.15	0.09		22
D1S1191	8.8	-infinity	-0.41	-0.17	-0.07	-0.02		0.33		0.33	0.2	0.11	0.06	0.02		23
D1S534	10.8	-infinity	-2.37	-1	-0.39	-0.1		-infinity		-infinity	-1.51	-0.67	-0.31	-0.12		24
D1S1653	7.1	-3.89	0.42	0.48	0.33	0.14		-2.74		-2.74	0.98	0.81	0.52	0.22		25
D1S1679	4	-infinity	-1.1	-0.33	-0.07	0.01		-infinity		-7.24	-0.63	-0.33	-0.2	-0.1		26
D1S1677	4															
D1S1625	11.6	-infinity	-0.21	-0.03	0.02	0.03		-infinity		0.44	0.45	0.31	0.17	0.06		27
D1S1589	10.3	-infinity	-0.71	-0.07	0.09	0.08		-infinity		-infinity	-0.32	0.08	0.11	0.05		28
D1S518	8.3	-infinity	-2.38	-1	-0.39	-0.1		-infinity		-infinity	-1.85	-0.87	-0.41	-0.16		29
D1S1660	6.3	-infinity	0.03	0.35	0.3	0.14		-infinity		-infinity	0.23	0.36	0.26	0.11		30
D1S1678	10.4	0.49	0.42	0.24	0.06	-0.03		0.43		0.43	0.23	0.04	-0.07	-0.06		31
GATA124F08	8.3	-infinity	0.53	0.76	0.59	0.29		-0.80		-0.8	1.54	1.2	0.75	0.32		32
D1S2141	14.3	-2.01	0.31	0.35	0.26	0.12		-2.31		-2.31	0.05	0.16	0.13	0.06		33
D1S549	11.2															
D1S3462	25.5	-infinity	-1.35	-0.47	-0.15	-0.03		-infinity		-infinity	-1.85	-0.86	-0.4	-0.15		34
D1S547	7.4	-2.33	0.55	0.64	0.5	0.28		-3.21		-1.44	0.69	0.61	0.43	0.21		35
D1S1609																

Table 13. Chromosome 2 two point lod score results

Marker name	Distance to next marker (cM)	MLINK analysis, affecteds and unaffecteds, V:7 classed as affected						GENEHUNTER analysis, affecteds only, V:7 classed as unknown		MLINK analysis, affecteds only, V:7 classed as affected						Multipoint graph locus number
		Recombination fraction								Recombination fraction						
		0	0.1	0.2	0.3	0.4				0	0.1	0.2	0.3	0.4		
GATA165C07	7.8	0.35	0.24	0.14	0.07	0.03		0.39		0.71	0.46	0.25	0.11	0.04		1
D2S1780	9.1	-2.1	0.43	0.43	0.32	0.17		-2.35		-2.35	0.21	0.26	0.2	0.11		2
GATA116B01	11.7	-infinity	0.79	0.63	0.4	0.18		1.07		-infinity	0.06	0.09	0.05	0.03		3
D2S1400	9.4	-infinity	0.13	0.32	0.26	0.13		-2.67		-2.37	0.26	0.23	0.14	0.05		4
D2S1360	10	-infinity	-0.2	0.17	0.19	0.11		-1.44		-1.14	0.97	0.81	0.53	0.24		5
D2S405	9.8	-infinity	0.16	0.31	0.2	0.05		-2.36		-1.82	0.36	0.32	0.19	0.06		6
D2S1788	5.7	-5.27	0.46	0.55	0.39	0.17		-6.49		-5.98	-0.11	0.12	0.12	0.06		7
D2S1346	5.8	-5.71	0.15	0.35	0.3	0.16		-6.61		-6.31	-0.35	-0.03	0.06	0.06		8
D2S1356	10.6	-6.52	-0.72	-0.32	-0.2	-0.13		-7.43		-7.13	-1.04	-0.46	-0.22	-0.09		9
D2S2739	4	-4.69	0.94	0.9	0.61	0.27		-5.91		-5.64	0.17	0.33	0.27	0.14		10
D2S1337	8.3	-infinity	-0.43	0.02	0.1	0.06		-1.75		-1.45	0.71	0.6	0.39	0.17		11
D2S441	11	-2.78	0.4	0.4	0.28	0.14		-3.10		-2.68	0.42	0.38	0.26	0.13		12
D2S1774	4.5	-infinity	-1.27	-0.75	-0.44	-0.2		-7.93		-7.93	-0.74	-0.34	-0.15	-0.05		13
D2S2733	5.2	-infinity	0.44	0.54	0.37	0.14		-infinity		-infinity	1.14	0.94	0.59	0.24		14
D2S1790	9.3	-infinity	-1.41	-0.64	-0.28	-0.1		-3.07		-infinity	-0.94	-0.39	-0.16	-0.04		15
GATA176C01	4.5	-infinity	-1.63	-0.65	-0.25	-0.07		-infinity		-infinity	-0.72	-0.19	-0.01	0.03		16
D2S436	8.5	-infinity	-1.03	-0.5	-0.28	-0.13		-infinity		-infinity	-1.43	-0.68	-0.31	-0.11		17
D2S410	8.7	-infinity	-1.35	-0.46	-0.13	-0.02		-infinity		-infinity	-1.41	-0.65	-0.31	-0.12		18

Marker name	Distance to next marker (cM)	MLINK analysis, affecteds and unaffecteds, V:7 classed as affected						GENEHUNTER analysis, affecteds only, V:7 classed as unknown		MLINK analysis, affecteds only, V:7 classed as affected						Multipoint graph locus number
		Recombination fraction								Recombination fraction						
		0	0.1	0.2	0.3	0.4				0	0.1	0.2	0.3	0.4		
D2S1328	11.5	-infinity	-1.2	-0.41	-0.09	0.02		-8.42		-8.42	-1.06	-0.44	-0.17	-0.05		19
D2S1334	4.6	-infinity	-0.9	-0.22	-0.04	-0.02		-infinity		-infinity	-1.72	-0.82	-0.4	-0.17		20
D2S442	7.2	-10.14	-0.77	-0.19	0.01	0.05		-10.44		-10.44	-1.03	-0.39	-0.13	-0.03		21
D2S1399	12.3	-infinity	-0.93	-0.38	-0.18	-0.09		-2.70		-6.8	-0.42	-0.13	-0.05	-0.03		22
D2S1353	13.3	-infinity	0.67	0.53	0.31	0.11		1.51		1.51	1.12	0.73	0.4	0.15		23
D2S1776	7	-infinity	-0.44	0.11	-0.01	0.01		-2.45		-infinity	-0.65	-0.25	-0.09	-0.02		24
GATA194A05	11.5	-infinity	0.5	0.54	0.37	0.16		-7.17		-infinity	-0.25	0.04	0.06	0.01		25
D2S1391	5.8	-infinity	-0.06	0.13	0.11	0.05		-infinity		-infinity	-0.54	-0.22	-0.12	-0.06		26
D2S2735	8.3															
D2S1384	12	-infinity	0.77	0.59	0.35	0.14		1.66		-infinity	0.53	0.43	0.25	0.1		27
D1S1649	6.3	-infinity	0.51	0.45	0.3	0.15		1.22		-infinity	0.22	0.25	0.18	0.09		28
D2S434	9.3	0.77	0.58	0.38	0.2	0.07		1.19		1.19	0.88	0.58	0.32	0.13		29
D2S1363	4.9	-infinity	-1.06	-0.41	-0.15	-0.04		-2.34		-infinity	-0.62	-0.22	-0.08	-0.02		30
D2S1370	7.2	1.15	1.03	0.74	0.43	0.17		1.67		1.56	1.16	0.76	0.41	0.15		31
D2S427	10.4	-infinity	-1.07	-0.52	-0.27	-0.11		1.13		-infinity	0.07	0.07	0.02	0		32
D2S1397	5.4	-infinity	-0.07	0.16	0.16	0.08		-2.34		-2.04	0.26	0.26	0.16	0.07		33
GATA178G09		-infinity	-1.21	-0.5	-0.19	-0.05		-2.78		-infinity	-0.72	-0.29	-0.12	-0.03		34

Table 14. Chromosome 3 two point lod score results

Marker name	Distance to next marker (cM)	MLINK analysis, affecteds and unaffecteds, V:7 classed as affected						GENEHUNTER analysis, affecteds only, V:7 classed as unknown		MLINK analysis, affecteds only, V:7 classed as affected						Multipoint graph locus number
		Recombination fraction								Recombination fraction						
		0	0.1	0.2	0.3	0.4				0	0.1	0.2	0.3	0.4		
D3S2387	8.5	-infinity	-2.13	-0.94	0.42	-0.15		-infinity		-infinity	-1.39	-0.58	-0.25	-0.09		1
D3S3050	5.6	-infinity	-0.08	0.22	0.2	0.1		-2.48		-2.48	0.36	0.36	0.19	0.07		2
D3S3030	10.2	-infinity	-0.13	0.12	0.14	0.09		-infinity		-infinity	-0.63	-0.25	-0.11	-0.04		3
GATA164B08	8.5	-infinity	-1.11	-0.47	-0.2	-0.07		-7.04		-6.74	-0.87	-0.39	-0.2	-0.09		4
D3S2403	8.4	-infinity	1.45	1.37	0.99	0.49		-2.39		-infinity	1.89	1.56	1.06	0.5		5
GATA140H01	4.5	-infinity	-0.67	-0.09	0.1	0.11		-infinity		-infinity	-1.04	-0.34	-0.06	0.03		6
D3S3038	4.9	-3.16	-0.31	-0.13	-0.05	-0.01		-3.16		-3.16	-0.31	-0.13	-0.05	-0.01		7
D3S2466	7.5	-infinity	-0.79	-0.26	-0.09	-0.03		-6.54		-infinity	-1.27	-0.49	-0.17	-0.03		8
D3S2432	2.8	-infinity	0.66	0.7	0.52	0.28		-1.99		-infinity	-0.08	0.14	0.16	0.1		9
D3S1768	11	-infinity	0.15	0.27	0.24	0.14		-infinity		-infinity	-0.11	0.06	0.09	0.07		10
D3S2409	7.6															
D3S1766	9.8	-infinity	-1.52	-0.64	-0.25	-0.06		-6.51		-infinity	-1.04	-0.42	-0.17	-0.05		11
GATA148E04	10.5	-infinity	-0.3	0.15	0.23	0.16		-infinity		-infinity	-0.55	-0.05	0.08	0.08		12
D3S2454	4.9	-infinity	-0.56	-0.21	-0.06	0		-infinity		-infinity	-0.79	-0.36	-0.15	-0.04		13
D3S2406	8.5	-infinity	-0.44	0.01	0.1	0.07		-infinity		-infinity	0.26	0.41	0.32	0.17		14
D3S4529	7.5	-infinity	-1.74	-0.67	-0.22	-0.03		-infinity		-infinity	-1.12	-0.34	-0.05	0.04		15
D3S2459	7	-infinity	-0.98	-0.32	-0.09	-0.01		-infinity		-infinity	-0.81	-0.22	-0.03	0.02		16
D3S3045	9	-infinity	-2.48	-0.106	-0.42	-0.11		-infinity		-infinity	-1.47	-0.54	-0.14	0.01		17

Marker name	Distance to next marker (cM)	MLINK analysis, affecteds and unaffecteds, V:7 classed as affected						GENEHUNTER analysis, affecteds only, V:7 classed as unknown		MLINK analysis, affecteds only, V:7 classed as affected						Multipoint graph locus number
		Recombination fraction								Recombination fraction						
		0	0.1	0.2	0.3	0.4				0	0.1	0.2	0.3	0.4		
D3S4018	5.7	-infinity	-2.07	-0.89	-0.35	-0.09		-infinity		-infinity	-1.09	-0.39	-0.09	0.02		18
D3S2460	4.4	-infinity	-1.23	-0.44	-0.13	-0.01		-infinity		-infinity	-0.76	-0.2	-0.01	0.04		19
ATA34G06	11	-infinity	-1.08	-0.42	-0.15	-0.03		-infinity		-infinity	-0.88	-0.31	-0.1	-0.02		20
D3S4527	8.6	-infinity	-0.88	-0.35	-0.13	-0.03		-6.61		-6.61	-0.46	-0.13	-0.03	-0.01		21
D3S1764	10.7	-infinity	0.16	0.31	0.24	0.12		-2.23		-infinity	-0.01	0.19	0.17	0.08		22
D3S1744	7.6	-infinity	-1.14	-0.45	-0.16	-0.03		-2.41		-infinity	-0.7	-0.26	-0.09	-0.01		23
D3S4531	6.4	-infinity	2.34	1.8	1.12	0.45		2.86		-infinity	1.53	1.18	0.71	0.28		24
D3S1763	6.2	-infinity	-1.49	-0.62	-0.24	-0.06		-6.36		-infinity	-0.54	-0.13	0.01	0.04		25
D3S3053	8.4	-infinity	-0.94	-0.27	-0.05	0.01		-3.46		-infinity	-0.57	-0.11	0.01	0.02		26
D3S2427	14.6	-infinity	-0.75	-0.09	0.09	0.09		-6.74		-infinity	-0.51	-0.03	0.08	0.07		27
D3S2436	5.9	0.39	0.27	0.16	0.08	0.02		0.33		0.69	0.47	0.29	0.14	0.05		28
D3S2398	5.2	-infinity	-0.65	-0.14	0	0.01		-7.16		-6.86	-0.26	0.1	0.16	0.11		29
D3S2418	9.5	1.41	1.04	0.7	0.4	0.16		0.81		1.11	0.79	0.5	0.27	0.1		30
D3S3054		-infinity	-0.77	-0.21	-0.02	0.01		-infinity		-infinity	-0.31	0.01	0.09	0.07		31

Table 15. Chromosome 4 two point lod score results

Marker name	Distance to next marker (cM)	MLINK analysis, affecteds and unaffecteds, V:7 classed as affected						GENEHUNTER analysis, affecteds only, V:7 classed as unknown		MLINK analysis, affecteds only, V:7 classed as affected						Multipoint graph locus number
		Recombination fraction								Recombination fraction						
		0	0.1	0.2	0.3	0.4				0	0.1	0.2	0.3	0.4		
D4S2366	16.7	-infinity	-1.43	-0.68	-0.33	-0.13		-7.17		-infinity	-0.89	-0.34	-0.13	-0.04		1
D4S2639	3.4	-infinity	0.67	0.69	0.49	0.24		-1.62		-1.59	0.9	0.73	0.47	0.21		2
GATA158G03	6.7	-infinity	-0.4	0.09	0.19	0.15		-2.32		-infinity	-0.08	0.16	0.17	0.1		3
D4S2397	3															
D4S3244	6.8	-infinity	0.08	0.11	0.07	0.03		-infinity		-infinity	-0.18	-0.09	-0.08	-0.05		4
D4S2632	13.5	-infinity	-0.54	-0.16	-0.03	0		-infinity		-infinity	-1	-0.48	-0.24	-0.1		5
D4S1627	6.7	-infinity	0.31	0.41	0.28	0.13		-infinity		-infinity	0.06	0.23	0.17	0.08		6
D4S2379	4.2	-infinity	-0.23	0.16	0.19	0.1		-infinity		-infinity	-0.84	-0.31	-0.13	-0.06		7
D4S1645	3.6	-infinity	-1.47	-0.64	-0.28	-0.1		-3.42		-infinity	-1.09	-0.55	-0.29	-0.12		8
D4S2432	3.9	-infinity	-0.93	-0.25	-0.03	0.03		-6.77		-infinity	-1.12	-0.44	-0.17	-0.05		9
D4S2367	5.1	-infinity	0.08	0.24	0.18	0.09		-infinity		-infinity	-0.41	-0.14	-0.07	-0.04		10
D4S3249	7.1															
D4S3243	3.6	-3.25	-0.77	-0.29	-0.09	-0.01		-2.85		-2.85	-0.56	-0.18	-0.04	0		11
D4S2361	9.6	-infinity	-1.94	-0.81	-0.31	-0.08		-infinity		-infinity	-1.85	-0.87	-0.39	-0.14		12
D4S2433	6.8	-infinity	-0.39	0.02	0.07	0.02		-infinity		-infinity	-1.11	-0.51	-0.25	-0.1		13
D4S1647	8.8	-infinity	-0.75	-0.27	-0.14	-0.09		-infinity		-infinity	-1.26	-0.62	-0.33	-0.15		14
D4S2623	16.8	-1.59	0.56	0.66	0.49	0.24		-2.50		-2.19	0.05	0.25	0.22	0.12		15
D4S2394	10.7	-10.35	-0.68	-0.1	0.08	0.1		-10.65		-10.65	-0.94	-0.31	-0.06	0.02		16

Marker name	Distance to next marker (cM)	MLINK analysis, affecteds and unaffecteds, V:7 classed as affected						GENEHUNTER analysis, affecteds only, V:7 classed as unknown		MLINK analysis, affecteds only, V:7 classed as affected						Multipoint graph locus number
		Recombination fraction								Recombination fraction						
		0	0.1	0.2	0.3	0.4				0	0.1	0.2	0.3	0.4		
D4S1644	5.8	-infinity	-0.84	-0.26	-0.06	0		-infinity		-infinity	-0.45	-0.11	-0.02	-0.01		17
D4S1625	18.1	-infinity	-0.94	-0.22	0	0.03		-infinity		-infinity	-0.85	-0.32	-0.13	-0.05		18
D4S1629	9	-infinity	-0.86	-0.25	-0.05	-0.01		-infinity		-infinity	-1.37	-0.63	-0.3	-0.12		19
D4S2368	7.5	-infinity	-0.67	-0.18	-0.03	0.01		-infinity		-infinity	-0.24	-0.01	0	-0.02		20
D4S2431	8.1	-infinity	-1.13	-0.51	-0.25	-0.1		-infinity		-infinity	-1.23	-0.59	-0.3	-0.13		21
D4S2417	8	-2.44	0.04	0.14	0.1	0.03		-3.04		-2.74	-0.22	-0.05	-0.01	0		22
D4S2374	12.1	-infinity	-2.45	-1.08	-0.48	-0.18		-infinity		-infinity	-1.54	-0.67	-0.29	-0.1		23
D4S3335	16.72	-infinity	-2.55	-1.31	-0.66	-0.26		-infinity		-infinity	-1.27	-0.62	-0.32	-0.14		24
D4S2390		-infinity	-0.97	-0.34	-0.11	-0.03		-2.86		-infinity	-0.59	-0.2	-0.07	-0.02		25

Table 16. Chromosome 5 two point lod score results

Marker name	Distance to next marker (cM)	MLINK analysis, affecteds and unaffecteds, V:7 classed as affected						GENEHUNTER analysis, affecteds only, V:7 classed as unknown		MLINK analysis, affecteds only, V:7 classed as affected						Multipoint graph locus number
		Recombination fraction								Recombination fraction						
		0	0.1	0.2	0.3	0.4				0	0.1	0.2	0.3	0.4		
D5S2488	4.9	-infinity	-0.98	-0.32	-0.08	0.01		-2.54		-2.24	-0.05	-0.03	-0.04	-0.02		1
GATA145D10	10.2	-infinity	-2.14	-0.78	-0.22	0		-infinity		-infinity	-2	-0.88	-0.37	-0.12		2
D5S2505	6.7	-infinity	-1.53	-0.65	-0.25	-0.06		-infinity		-infinity	-1.13	-0.54	-0.27	-0.11		3
D5S807	5	-infinity	-1.02	-0.31	-0.08	-0.01		-infinity		-infinity	-1	-0.42	-0.19	-0.08		4
D5S817	16.7	1.05	0.78	0.52	0.28	0.09		0.45		0.75	0.53	0.33	0.16	0.05		5
D5S1473	4.7	-infinity	-1.81	-0.78	-0.3	-0.08		-infinity		-infinity	-1.23	-0.57	-0.27	-0.1		6
GATA145D09	7.4	0.67	0.54	0.38	0.23	0.09		-1.29		0.07	0.03	-0.01	-0.03	-0.02		28
D5S1470	5.1	-infinity	0.51	0.54	0.35	0.14		1.87		2.17	1.63	1.1	0.61	0.23		7
D5S2843	10.2	-infinity	-0.16	0.07	0.07	0.02		-2.83		-2.53	-0.06	0.06	0.05	0.01		8
D5S1457	9.4	-infinity	-1.07	-0.51	-0.29	-0.15		-infinity		-infinity	-0.62	-0.3	-0.17	-0.08		9
D5S2507	3.5	-infinity	-1.16	-0.41	-0.14	-0.05		-infinity		-infinity	-0.27	0.01	0.05	0.03		10
D5S2500	18.6	-infinity	-1.6	-0.7	-0.32	-0.13		-infinity		-infinity	-1.15	-0.5	-0.22	-0.08		11
D5S806	13.7	-1.51	0.99	0.8	0.52	0.23		-2.65		-2.04	0.54	0.45	0.29	0.13		12
D5S1459	3.2	-2.67	-0.25	-0.07	-0.02	0		-2.67		-2.67	-0.25	-0.07	-0.02	0		13
D5S1725	6.8	-6.06	-0.26	0.08	0.14	0.07		-6.96		-6.66	-0.77	-0.31	-0.13	-0.05		14
D5S1462	8.3	-1.47	0.8	0.66	0.41	0.16		-1.77		-1.77	0.54	0.45	0.26	0.08		15
D5S1453	2.3	-infinity	0.22	0.21	0.13	0.06		0.96		0.96	0.61	0.33	0.13	0.03		16
D5S2501	5.3	-infinity	-1.94	-0.72	-0.25	-0.06		-8.91		-8.61	-1.38	-0.57	-0.25	-0.11		17

Marker name	Distance to next marker (cM)	MLINK analysis, affecteds and unaffecteds, V:7 classed as affected						GENEHUNTER analysis, affecteds only, V:7 classed as unknown		MLINK analysis, affecteds only, V:7 classed as affected						Multipoint graph locus number
		Recombination fraction								Recombination fraction						
		0	0.1	0.2	0.3	0.4				0	0.1	0.2	0.3	0.4		
GATA130E01	5.6	-infinity	0.2	0.35	0.28	0.15		-2.21		-1.72	0.63	0.52	0.31	0.13		18
D5S1505	4.1	-infinity	-0.16	0.2	0.19	0.08		-6.01		-5.7	-0.01	0.15	0.1	0.02		19
D5S1495	6.6	-infinity	0.69	0.55	0.35	0.15		1.26		1.56	1.12	0.71	0.38	0.14		20
D5S816	9.5	-infinity	-0.29	-0.03	0.02	0.01		-2.54		-2.24	0.36	0.29	0.15	0.04		21
D5S1480	12.8	-2.95	-0.13	0	0.02	0.01		-2.99		-2.99	-0.18	-0.04	-0.01	-0.01		22
D5S820	6.7	-infinity	-0.17	0.28	0.32	0.19		-1.92		-1.62	0.41	0.55	0.43	0.22		23
D5S1465	7.6	-infinity	-1.36	-0.54	-0.2	-0.05		-2.49		-2.19	0.28	0.24	0.12	0.03		24
D5S1471	4.7	-infinity	1.87	1.51	1	0.46		3.18		3.18	2.56	1.91	1.22	0.55		25
D5S1456	2.4	-infinity	-0.79	-0.23	-0.04	0.02		0.79		-2.83	-0.06	0.02	0	-0.02		26
D5S1713		-infinity	1.37	1.08	0.68	0.3		2.63		2.63	2.06	1.47	0.89	0.38		27

Table 17. Chromosome 6 two point lod score results

Marker name	Distance to next marker (cM)	MLINK analysis, affecteds and unaffecteds, V:7 classed as affected						GENEHUNTER analysis, affecteds only, V:7 classed as unknown		MLINK analysis, affecteds only, V:7 classed as affected						Multipoint graph locus number
		Recombination fraction								Recombination fraction						
		0	0.1	0.2	0.3	0.4				0	0.1	0.2	0.3	0.4		
D6S1955	8.6	-7.37	-1.11	-0.57	-0.3	-0.13		-7.27		-7.27	-0.57	-0.21	-0.08	-0.02		1
D6S1279	6.6	-6.75	-0.56	-0.14	-0.02	0		-6.21		-6.51	-0.16	0.09	0.11	0.06		2
D6S1034	12.4	-6.5	-0.49	-0.12	-0.02	-0.01		-6.29		-6.29	-0.41	-0.08	0.01	0.02		3
D6S1959	3.1															
D6S1266	8.3	-infinity	-1.02	-0.4	-0.14	-0.03		-6.91		-6.91	-0.32	0	0.08	0.07		4
GATA163B10	5.8	-infinity	-1.75	-0.66	-0.2	-0.02		-infinity		-infinity	-0.37	0.11	0.21	0.15		5
D9S1126	4.8	-infinity	1.01	0.96	0.67	0.31		-0.98		-infinity	0.25	0.38	0.28	0.13		6
GGAA15B08	8	-infinity	-0.79	-0.26	-0.09	-0.04		-6.15		-6.15	-0.35	-0.06	0.01	0.01		7
D6S1017	13.6	-infinity	-1.6	-0.98	-0.69	-0.38		-2.30		-6.98	-0.48	-0.17	-0.08	-0.05		8
GATA11E02	8.8	-8.55	-1.37	-0.55	-0.19	-0.03		-6.12		-6.12	-0.13	0.17	0.2	0.12		9
D6S1053	6.4	-10.78	-1.29	-0.56	-0.24	-0.09		-11.38		-11.08	-1.24	-0.58	-0.29	-0.12		10
D6S1031	5.6	-infinity	0.17	0.36	0.31	0.18		-infinity		-infinity	-0.05	0.18	0.19	0.11		11
D6S1270	7.5	0.57	0.38	0.22	0.1	0.03		0.57		0.57	0.38	0.22	0.1	0.03		12
D6S1043	3.3	-infinity	-0.24	0.01	0.04	0.02		-infinity		-infinity	0.31	0.28	0.17	0.07		13
D6S1056	2.7	-infinity	-0.62	-0.13	0.02	0.05		-infinity		-infinity	-0.66	-0.13	0.04	0.06		14
D6S1284	5.8	-2.87	-0.02	0.09	0.07	0.04		-2.37		-2.34	0.34	0.32	0.21	0.1		15
D6S1021	10.3	-infinity	0.61	0.49	0.3	0.12		-2.97		-2.79	0.42	0.34	0.19	0.07		16
D6S474	8.1	-infinity	-0.44	-0.03	0.03	0.01		-infinity		-infinity	-0.47	-0.16	-0.06	-0.02		17

Marker name	Distance to next marker (cM)	MLINK analysis, affecteds and unaffecteds, V:7 classed as affected						GENEHUNTER analysis, affecteds only, V:7 classed as unknown		MLINK analysis, affecteds only, V:7 classed as affected						Multipoint graph locus number
		Recombination fraction								Recombination fraction						
		0	0.1	0.2	0.3	0.4				0	0.1	0.2	0.3	0.4		
D6S1958	3	0.51	0.46	0.32	0.18	0.07		0.98		0.98	0.7	0.44	0.22	0.08		18
D6S1040	7.2	-infinity	-1.21	-0.45	-0.17	-0.05		-infinity		-infinity	-0.4	-0.11	-0.04	-0.02		19
D6S1009	7.3	-infinity	-0.52	-0.09	0.01	0.02		-infinity		-infinity	-0.27	-0.01	0.04	0.02		20
D6S1003	2.2	-3.38	-0.39	-0.15	-0.05	-0.01		-2.96		-2.96	-0.17	-0.04	-0.01	0		21
GATA184A08	9.1	-infinity	-1.95	-0.82	-0.32	-0.08		-infinity		-infinity	-0.15	0.09	0.11	0.07		22
GATA165G02	13.8	-infinity	-0.32	0.24	0.29	0.17		-infinity		-7.22	0.85	0.89	0.66	0.33		23
D6S1008	3.9	-infinity	-0.49	-0.01	0.1	0.07		-infinity		-6.51	-0.44	-0.08	0.01	0.01		24
D6S1273	3.1	-infinity	-0.82	-0.34	-0.13	-0.03		-infinity		-infinity	-0.99	-0.43	-0.17	-0.04		25
D6S1277	8.5	-infinity	-1.56	-0.73	-0.34	-0.13		-infinity		-3.23	-0.46	-0.28	-0.16	-0.08		26
D6S392	9.5	-infinity	-0.53	-0.09	0.01	0.03		-infinity		-1.66	0.86	0.69	0.43	0.18		27
D6S1027		-infinity	-0.99	-0.28	-0.06	0.01		-infinity		-infinity	-0.62	-0.15	-0.01	0.02		28

Table 18. Chromosome 7 two point lod score results

Marker name	Distance to next marker (cM)	MLINK analysis, affecteds and unaffecteds, V:7 classed as affected						GENEHUNTER analysis, affecteds only, V:7 classed as unknown		MLINK analysis, affecteds only, V:7 classed as affected						Multipoint graph locus number
		Recombination fraction								Recombination fraction						
		0	0.1	0.2	0.3	0.4				0	0.1	0.2	0.3	0.4		
D7S1819	6.7	-1.72	0.51	0.47	0.33	0.16		-2.02		-2.02	0.26	0.27	0.18	0.08		1
D7S2201	8	-3.05	-0.36	-0.18	-0.11	-0.06		-2.83		-2.83	-0.03	0.05	0.04	0.02		2
D7S3047	14.4															
GATA137H02	3.1	-infinity	-0.59	-0.1	0.03	0.03		-6.70		-6.4	-0.46	-0.1	-0.01	0		3
D7S1802	8.2	-infinity	0.03	0.16	0.11	0.05		-2.93		-2.9	0.53	0.41	0.23	0.08		4
GATA124D01	9.5	-infinity	-1.53	-0.55	-0.19	-0.05		-5.69		-5.39	0.25	0.36	0.25	0.1		5
D7S1808	6.6	-infinity	-1.12	-0.51	-0.27	-0.14		-3.37		-3.07	-0.18	-0.03	-0.02	-0.04		6
D7S817	7.9	-infinity	-2.55	-0.131	-0.68	-0.28		-infinity		-infinity	-1.43	-0.76	-0.43	-0.2		7
D7S2846	5.4	-infinity	-1.17	-0.42	-0.15	-0.04		-infinity		-infinity	-0.09	0.1	0.09	0.05		8
D7S3043	11.3	-infinity	-1.69	-0.8	-0.39	-0.16		-infinity		-infinity	-1.3	-0.62	-0.3	-0.12		9
D7S1818	9.5	-infinity	-0.08	0.2	0.23	0.15		-2.78		-infinity	-0.78	-0.3	-0.11	-0.03		10
GATA118G10	7.8	-infinity	-1.89	-0.79	-0.31	-0.09		-infinity		-infinity	-2.09	-0.96	-0.44	-0.16		11
D7S1816	5.7	-infinity	-1.76	-0.89	-0.45	-0.17		-infinity		-infinity	-1.74	-0.8	-0.36	-0.13		12
D7S2204	8.3	-2.6	0.07	0.14	0.1	0.05		-2.24		-2.24	0.33	0.32	0.21	0.1		13
D7S820	6.5	-infinity	-2.88	-1.4	-0.68	-0.26		-infinity		-infinity	-1.43	-0.62	-0.28	-0.11		14
D7S1813	6.2	-infinity	-2.14	-1.1	-0.57	-0.23		-infinity		-infinity	-1.44	-0.71	-0.35	-0.13		15
D7S821	5.5	-infinity	-1.69	-0.96	-0.61	-0.34		-infinity		-infinity	-1.19	-0.52	-0.26	-0.15		16
D7S1799	8.8	-infinity	-1.16	-0.5	-0.22	-0.08		-2.45		-infinity	-0.73	-0.3	-0.12	-0.04		17

Marker name	Distance to next marker (cM)	MLINK analysis, affecteds and unaffecteds, V:7 classed as affected						GENEHUNTER analysis, affecteds only, V:7 classed as unknown		MLINK analysis, affecteds only, V:7 classed as affected						Multipoint graph locus number
		Recombination fraction								Recombination fraction						
		0	0.1	0.2	0.3	0.4				0	0.1	0.2	0.3	0.4		
D7S1817	7.4	-infinity	-0.12	0.12	0.12	0.05		-2.62		-2.32	0.19	0.2	0.12	0.04		18
GGAA6D03	10.5	-infinity	0.78	0.66	0.41	0.17		-infinity		-infinity	0.89	0.71	0.43	0.18		19
D7S1804	6.7	-infinity	-2.99	-1.48	-0.72	-0.28		-infinity		-infinity	-1.63	-0.75	-0.36	-0.14		20
D7S1837	12.1	-infinity	-0.92	-0.36	-0.15	-0.06		-5.81		-5.81	-0.22	0.03	0.07	0.04		21
D7S1824	5.1	-infinity	-2.38	-1.15	-0.55	-0.2		-infinity		-infinity	-1.23	-0.56	-0.25	-0.09		22
D7S2195	3.4	-infinity	-0.98	-0.29	-0.09	-0.05		-infinity		-infinity	0.38	0.47	0.32	0.12		23
D7S2208	9.3	-infinity	-0.83	-0.34	-0.19	-0.11		-infinity		-infinity	-0.95	-0.43	-0.22	-0.1		24
D7S1798	1.4	-infinity	-0.69	-0.28	-0.1	-0.02		-2.98		-infinity	-0.68	-0.27	-0.1	-0.02		25
GATA189C06	8.2	-infinity	-0.91	-0.35	-0.14	-0.05		-infinity		-infinity	-0.55	-0.19	-0.06	-0.02		26
D7S1807	4															
D7S1823		-infinity	-1.93	-0.8	-0.29	-0.07		-3.05		-infinity	-1.34	-0.55	-0.2	-0.04		27

Table 19. Chromosome 8 two point lod score results

Marker name	Distance to next marker (cM)	MLINK analysis, affecteds and unaffecteds, V:7 classed as affected						GENEHUNTER analysis, affecteds only, V:7 classed as unknown		MLINK analysis, affecteds only, V:7 classed as affected						Multipoint graph locus number
		Recombination fraction								Recombination fraction						
		0	0.1	0.2	0.3	0.4				0	0.1	0.2	0.3	0.4		
D8S1469	9	-infinity	0.18	0.36	0.31	0.17		-1.86		-infinity	-0.29	0.01	0.07	0.05		1
D8S1130	5.6	-infinity	0.4	0.5	0.37	0.19		-1.44		-infinity	-0.01	0.19	0.18	0.09		2
D8S1106	6	-infinity	0.58	0.62	0.42	0.17		-1.64		-infinity	-0.09	0.14	0.13	0.06		3
D8S1145	15.4	0.94	0.67	0.43	0.24	0.09		0.68		0.69	0.44	0.24	0.1	0.03		4
D8S1989	14.1	-infinity	0.26	0.33	0.22	0.1		-2.60		-infinity	-0.24	0.02	0.05	0.03		5
D8S1477	4.3	-infinity	0.17	0.31	0.23	0.1		-infinity		-infinity	-0.09	0.11	0.09	0.03		6
D8S1104	2.9	-1.85	0.74	0.64	0.43	0.2		-2.46		-2.16	0.49	0.44	0.3	0.14		7
D8S1110	7.1															
D8S593	6															
D8S1113	7.3	-infinity	0.47	0.53	0.36	0.15		-2.20		-1.9	0.65	0.51	0.29	0.11		8
D8S1136	4.8	-3.32	-0.41	-0.09	-0.03	-0.02		-1.93		-2.67	-0.21	0.04	0.07	0.03		9
D8S2323	8	-infinity	-0.5	-0.12	-0.05	-0.04		-2.56		-2.26	-0.23	0.02	0.05	0.03		10
GATA14E09	4.9	-infinity	-1.34	-0.66	-0.33	-0.14		-infinity		-infinity	-0.55	-0.2	-0.07	-0.02		11
D8S1475	5.6															
D8S1119	4.5	-infinity	-1.05	-0.48	-0.25	-0.11		-infinity		-infinity	-0.31	-0.07	-0.04	-0.03		12
D8S1988	5.5	-2.12	0.51	0.45	0.29	0.13		-2.72		-2.42	0.26	0.25	0.16	0.07		13
GAAT1A4	9.5	-infinity	-0.78	-0.28	-0.13	-0.06		-infinity		-infinity	-1.09	-0.45	-0.18	-0.05		14
D8S1459	4.4	1.51	1.13	0.76	0.43	0.17		0.92		1.22	0.88	0.57	0.31	0.12		15

Marker name	Distance to next marker (cM)	MLINK analysis, affecteds and unaffecteds, V:7 classed as affected						GENEHUNTER analysis, affecteds only, V:7 classed as unknown		MLINK analysis, affecteds only, V:7 classed as affected						Multipoint graph locus number
		Recombination fraction								Recombination fraction						
		0	0.1	0.2	0.3	0.4				0	0.1	0.2	0.3	0.4		
D8S1470	2	-infinity	-0.08	0.26	0.23	0.11		-infinity		-infinity	0.37	0.46	0.32	0.14		16
D8S1471	1.3	-infinity	-1.92	-0.91	-0.44	-0.17		-infinity		-infinity	-0.97	-0.39	-0.17	-0.06		17
D8S592	3	-infinity	-1.39	-0.6	-0.3	-0.15		-infinity		-infinity	-0.94	-0.39	-0.19	-0.09		18
D8S1142	3.5															
D8S586	10.9	-infinity	-1.35	-0.72	-0.38	-0.15		-infinity		-infinity	-0.5	-0.2	-0.1	-0.05		19
D8S568	3.1	-9.96	-0.69	-0.23	-0.12	-0.07		-10.73		-10.43	-1.02	-0.43	-0.19	-0.07		20
D8S1179	5.5	-infinity	-2.41	-1.23	-0.67	-0.31		-infinity		-infinity	-1.93	-0.97	-0.5	-0.21		21
D8S1128	4	-infinity	-0.33	-0.11	-0.05	-0.03		-infinity		-infinity	-0.2	0.01	0.03	0.02		22
D8S1985	6	-infinity	1.02	0.77	0.44	0.16		1.68		-infinity	0.53	0.41	0.22	0.08		23
D8S1462	7.1	-infinity	-0.73	-0.3	-0.12	-0.03		-2.86		-2.86	-0.03	0.1	0.11	0.07		24
D8S1100		1.16	0.83	0.54	0.3	0.12		0.10		0.76	0.5	0.3	0.15	0.06		25

Table 20. Chromosome 9 two point lod score results

Marker name	Distance to next marker (cM)	MLINK analysis, affecteds and unaffecteds, V:7 classed as affected						GENEHUNTER analysis, affecteds only, V:7 classed as unknown		MLINK analysis, affecteds only, V:7 classed as affected						Multipoint graph locus number
		Recombination fraction								Recombination fraction						
		0	0.1	0.2	0.3	0.4				0	0.1	0.2	0.3	0.4		
GATA62F03	9.7	-infinity	-0.32	-0.04	0.02	0.02		-2.67		-2.55	-0.08	0.01	0.01	0		1
D9S921	12.4	-infinity	-0.88	-0.08	0.15	0.14		-infinity		-infinity	-0.03	0.35	0.35	0.2		2
D9S925	12.4	-infinity	0.66	0.81	0.61	0.29		-2.04		-infinity	0.78	0.79	0.54	0.24		3
D9S932	3.8	-infinity	-1.74	-0.74	-0.28	-0.07		-infinity		-infinity	-1.81	-0.85	-0.39	-0.13		4
D9S1121	9	-5.98	0.02	0.19	0.14	0.05		-1.90		-1.85	0.31	0.27	0.15	0.04		5
D9S304	1.7	-infinity	-2.06	-0.87	-0.33	-0.08		-infinity		-infinity	-1.61	-0.73	-0.32	-0.11		6
D9S1118	9.2	-infinity	-2.05	-0.9	-0.37	-0.11		-infinity		-infinity	-1.88	-0.92	-0.46	-0.18		7
D9S301	1.4	-infinity	-1.09	-0.51	-0.24	-0.09		-2.71		-infinity	-0.51	-0.25	-0.15	-0.08		8
D9S1124	9.2	-10.73	-1.09	-0.39	-0.1	-0.01		-10.59		-10.59	-1.09	-0.43	-0.15	-0.03		9
D9S1122	4.6	-infinity	-1.21	-0.54	-0.24	-0.09		-6.86		-infinity	-0.75	-0.3	-0.11	-0.03		10
D9S922	1.1	-infinity	-0.52	-0.04	0.09	0.08		-1.81		-infinity	-0.28	0	0.05	0.03		11
D9S303	3.2	-infinity	-1.04	-0.4	-0.14	-0.03		-infinity		-infinity	-1.5	-0.74	-0.36	-0.14		12
D9S1119	7.7	-infinity	-0.37	-0.09	0	0.02		-infinity		-infinity	-0.85	-0.44	-0.23	-0.1		13
D9S252	7.3	-infinity	-1.22	-0.48	-0.18	-0.05		-6.71		-infinity	-0.63	-0.25	-0.11	-0.04		14
D9S906	9.2	-infinity	-0.28	-0.16	-0.11	-0.06		-infinity		-infinity	-0.28	-0.16	-0.11	-0.06		15
D9S910	5.3	-infinity	-0.8	-0.22	-0.01	0.05		-2.90		-infinity	-0.74	-0.35	-0.17	-0.07		16
D9S938	8.3	-infinity	-0.25	0.02	0.07	0.05		-infinity		-infinity	-0.5	-0.18	-0.08	-0.03		17
D9S2026	1.1	-infinity	-1.84	-0.85	-0.4	-0.16		-infinity		-infinity	-1.83	-0.87	-0.42	-0.15		18

Marker name	Distance to next marker (cM)	MLINK analysis, affecteds and unaffecteds, V:7 classed as affected						GENEHUNTER analysis, affecteds only, V:7 classed as unknown		MLINK analysis, affecteds only, V:7 classed as affected						Multipoint graph locus number
		Recombination fraction								Recombination fraction						
		0	0.1	0.2	0.3	0.4				0	0.1	0.2	0.3	0.4		
D9S930	3.7	-infinity	0.7	0.73	0.52	0.25		-2.55		-infinity	-0.05	0.15	0.13	0.06		19
D9S907	2.8	-infinity	0.3	0.6	0.52	0.28		-5.78		-infinity	-0.75	-0.23	-0.06	0		20
D9S934	2.9	-infinity	-0.59	-0.27	-0.17	-0.11		-infinity		-infinity	-0.76	-0.37	-0.2	-0.09		21
D9S302	7.4	-infinity	-1.07	-0.28	0	0.07		-7.07		-infinity	-0.97	-0.39	-0.16	-0.05		22
D9S918	6.8	-6.8	-0.78	-0.33	-0.13	-0.04		-6.65		-6.65	-0.67	-0.25	-0.09	-0.02		23
D9S752	6.9	-infinity	0.19	0.34	0.26	0.13		-2.26		-1.95	0.61	0.5	0.3	0.12		24
ATA59H06	2.6															
D9S915		-3.44	-0.28	-0.04	0.03	0.03		-0.93		0.03	-0.02	-0.02	0.01	0.01		25

Table 21. Chromosome 10 two point lod score results

Marker name	Distance to next marker (cM)	MLINK analysis, affecteds and unaffecteds, V:7 classed as affected						GENEHUNTER analysis, affecteds only, V:7 classed as unknown		MLINK analysis, affecteds only, V:7 classed as affected						Multipoint graph locus number
		Recombination fraction								Recombination fraction						
		0	0.1	0.2	0.3	0.4				0	0.1	0.2	0.3	0.4		
D10S1435	10.3															
D10S1415	11.4															
D10S1412	6.7	-infinity	-0.69	-0.14	0.04	0.06		-3.17		-3.17	-0.72	-0.33	-0.16	-0.07		1
D10S2325	7.1	-infinity	-1.61	-0.72	-0.31	-0.09		-6.83		-infinity	-1.03	-0.45	-0.2	-0.08		2
D10S674	3.6	-infinity	-0.45	-0.13	-0.03	0		-3.31		-3.31	-0.47	-0.23	-0.14	-0.07		3
D10S1423	8.8	-2.19	0.37	0.33	0.21	0.08		-2.21		-2.22	0.35	0.32	0.2	0.08		4
D10S1214	4.3	-infinity	-1.05	-0.45	-0.2	-0.08		-infinity		-infinity	-1.29	-0.63	-0.32	-0.13		5
D10S1426	4.4	-infinity	-1.63	-0.75	-0.37	-0.16		-infinity		-infinity	-1.42	-0.7	-0.35	-0.14		6
D10S1208	6.9	-infinity	-0.99	-0.22	0.04	0.08		-infinity		-infinity	-0.99	-0.38	-0.12	-0.02		7
D10S1220	8.3															
D10S1221	1.5	-infinity	-0.73	-0.26	-0.12	-0.06		-infinity		-infinity	0.41	0.33	0.17	0.05		8
D10S1227	4	-infinity	-0.95	-0.42	-0.22	-0.1		-infinity		-infinity	0.19	0.17	0.07	0.01		9
D10S1428	7.6	-infinity	-1.53	-1.02	-0.6	-0.26		-2.73		-infinity	-0.75	-0.4	-0.23	-0.1		10
GATA121A08	8.2	-infinity	-0.85	-0.33	-0.14	-0.04		-infinity		-infinity	-1.11	-0.48	-0.2	-0.06		11
D10S1432	6.7	-infinity	-1.05	-0.41	-0.14	-0.02		-infinity		-infinity	-0.96	-0.33	-0.08	0.01		12
D10S2327	3.3															
D10S1427	9	-infinity	1.24	1.06	0.69	0.3		-2.39		-infinity	1.51	1.22	0.79	0.35		13
GATA115E01	3.6															

Marker name	Distance to next marker (cM)	MLINK analysis, affecteds and unaffecteds, V:7 classed as affected						GENEHUNTER analysis, affecteds only, V:7 classed as unknown		MLINK analysis, affecteds only, V:7 classed as affected						Multipoint graph locus number
		Recombination fraction								Recombination fraction						
		0	0.1	0.2	0.3	0.4				0	0.1	0.2	0.3	0.4		
D10S677	10															
D10S1239	2.9	-infinity	-0.71	-0.2	-0.04	0		0.06		-infinity	-0.54	-0.18	-0.04	0		14
D10S1246	6	-1.96	0.5	0.43	0.26	0.1		-1.97		-1.97	0.49	0.42	0.26	0.1		15
D10S1237	8.3	-infinity	-0.82	-0.27	-0.08	-0.02		-2.00		-infinity	-0.38	-0.06	0.02	0.02		16
D10S1236	2	-infinity	-0.28	-0.11	-0.03	0		-2.66		-infinity	-0.5	-0.19	-0.06	-0.01		17
D10S1230	4.9	-2.43	0.28	0.32	0.25	0.14		0.24		-3.59	-0.3	-0.1	-0.04	-0.01		18
D10S1213	1.3	-infinity	0.76	0.64	0.44	0.22		1.04		-infinity	0.06	0.1	0.07	0.03		19
D10S2322	6.7	-infinity	-0.4	0.05	0.15	0.12		-2.24		-infinity	-0.43	-0.13	-0.04	-0.01		20
D10S1223	5.7	-infinity	-1.56	-0.73	-0.3	-0.08		-2.95		-infinity	-0.62	-0.24	-0.09	-0.02		21
D10S1134	6	-infinity	-0.91	-0.35	-0.15	-0.06		-infinity		-infinity	-1.22	-0.58	-0.29	-0.12		22
D10S1248		-infinity	-0.85	-0.35	-0.13	-0.02		-2.55		-2.25	0.28	0.29	0.21	0.1		23

Table 22. Chromosome 11 two point lod score results

Marker name	Distance to next marker (cM)	MLINK analysis, affecteds and unaffecteds, V:7 classed as affected						GENEHUNTER analysis, affecteds only, V:7 classed as unknown		MLINK analysis, affecteds only, V:7 classed as affected						Multipoint graph locus number
		Recombination fraction								Recombination fraction						
		0	0.1	0.2	0.3	0.4				0	0.1	0.2	0.3	0.4		
D11S1984	4.8	-infinity	-1.46	-0.77	-0.42	-0.19		-infinity		-infinity	-0.87	-0.43	-0.23	-0.1		1
D11S2362	6.8	-2.91	-0.53	-0.34	-0.2	-0.09		-2.62		-2.62	-0.17	-0.04	-0.02	-0.01		2
D11S1999	10.1	-infinity	-1.24	-0.57	-0.25	-0.07		-6.85		-infinity	-1.27	-0.54	-0.21	-0.05		3
D11S1981	7.6	-infinity	-0.74	-0.26	-0.09	-0.02		0.90		-3.17	0.04	0.1	0.06	0.02		4
ATA34E08	8.6	-6.82	-0.76	-0.36	-0.21	-0.12		-6.99		-6.69	-0.64	-0.25	-0.11	-0.04		5
D11S1392	12.4	-infinity	-1.67	-0.88	-0.45	-0.18		-infinity		-infinity	-1.39	-0.69	-0.33	-0.12		6
D11S1993	4.8	-infinity	-1.2	-0.33	-0.03	0.04		-1.40		-infinity	0.39	0.53	0.41	0.21		7
D11S2019	10.1															
D11S2371	10.4	-infinity	-1.07	-0.54	-0.29	-0.13		-0.35		-infinity	-0.67	-0.31	-0.15	-0.06		8
D11S4960	3.5	-infinity	-2.16	-1.08	-0.54	-0.21		-infinity		-infinity	-1.33	-0.65	-0.31	-0.11		9
D11S1979	5.3	-infinity	-1.08	-0.42	-0.14	-0.02		-2.58		-infinity	-0.34	-0.07	0.01	0.02		10
D11S4952	8.2	-infinity	-2.32	-1.2	-0.61	-0.24		-infinity		-infinity	-2.32	-1.2	-0.61	-0.24		11
D11S2000	6.1	-infinity	-1.33	-0.67	-0.32	-0.11		-10.87		-infinity	-1.3	-0.57	-0.24	-0.07		12
D11S1986	9															
D11S1998	10	-infinity	-0.24	-0.12	-0.08	-0.04		0.32		0.62	0.41	0.23	0.09	0.02		13
D11S4464	11.8	-infinity	-1.24	-0.51	-0.2	-0.05		-6.47		-infinity	-1.24	-0.51	-0.2	-0.05		14
D11S4463	11.3	-infinity	-0.73	-0.13	0.07	0.09		-3.00		-infinity	-0.83	-0.32	-0.11	-0.02		15
D11S2359		1.7	1.34	0.97	0.59	0.25		1.87		1.86	1.47	1.06	0.65	0.28		16

Table 23. Chromosome 12 two point lod score results

Marker name	Distance to next marker (cM)	MLINK analysis, affecteds and unaffecteds, V:7 classed as affected						GENEHUNTER analysis, affecteds only, V:7 classed as unknown		MLINK analysis, affecteds only, V:7 classed as affected						Multipoint graph locus number
		Recombination fraction								Recombination fraction						
		0	0.1	0.2	0.3	0.4				0	0.1	0.2	0.3	0.4		
D12S372	9.5	-2.13	0.5	0.44	0.28	0.12		-2.31		-2.32	0.32	0.28	0.17	0.07		1
D12S374	5.4	-infinity	-0.96	-0.55	-0.35	-0.18		-infinity		-infinity	-0.88	-0.48	-0.26	-0.11		2
D3S2395	8.2															
D12S391	9.6	-infinity	-0.17	0.18	0.21	0.12		-1.64		-1.26	0.83	0.7	0.46	0.21		3
D12S373	7.2	-infinity	-0.55	-0.15	-0.01	0.02		-infinity		-infinity	-0.43	-0.08	0.03	0.04		4
D12S1066	7	-6.9	-0.76	-0.34	-0.16	-0.06		-6.76		-6.76	-0.66	-0.26	-0.11	-0.04		5
D12S1042	10.6	-6.67	-0.43	-0.11	-0.05	-0.06		-3.46		-3.16	-0.3	-0.08	-0.02	-0.01		6
D12S1301	7.3	-infinity	-0.96	-0.41	-0.18	-0.06		-2.11		-1.81	0.52	0.4	0.2	0.05		7
D12S2196	7.4	-infinity	-1.02	-0.43	-0.18	-0.05		-2.57		-2.57	0.1	0.11	0.05	0.01		8
D12S398	5.1	-infinity	0.04	0.04	-0.01	-0.02		-0.22		0.57	0.35	0.2	0.09	0.03		9
D12S1056	4.2	-2.93	-0.3	-0.11	-0.03	0		-3.28		-3.08	-0.34	-0.1	-0.01	0.01		10
D12S1294	5.2	-infinity	-0.84	-0.25	-0.05	0		-7.08		-6.78	-0.47	-0.13	-0.03	-0.01		11
D12S375	5.2	-infinity	-1.89	-0.95	-0.48	-0.2		-infinity		-infinity	-0.5	-0.17	-0.07	-0.03		12
D12S1052	9	-infinity	-1.38	-0.58	-0.26	-0.11		-3.33		-3.03	-0.46	-0.13	-0.04	-0.02		13
D12S2068	3.8	-infinity	-0.74	-0.29	-0.1	-0.02		-2.83		-2.53	0.05	0.13	0.09	0.04		14
D12S1064	6.7	-infinity	-0.83	-0.46	-0.3	-0.17		-3.60		-2.61	-0.14	-0.15	-0.19	-0.15		15
D12S1044	7.4	-infinity	-0.74	-0.3	-0.11	-0.02		-2.68		-2.57	0.11	0.14	0.09	0.04		16
D12S1051	7.1	-infinity	-0.67	-0.04	0.12	0.1		-1.75		-1.45	0.38	0.49	0.37	0.19		17

Marker name	Distance to next marker (cM)	MLINK analysis, affecteds and unaffecteds, V:7 classed as affected						GENEHUNTER analysis, affecteds only, V:7 classed as unknown		MLINK analysis, affecteds only, V:7 classed as affected						Multipoint graph locus number
		Recombination fraction								Recombination fraction						
		0	0.1	0.2	0.3	0.4				0	0.1	0.2	0.3	0.4		
PAH	18.3	-infinity	-0.53	-0.16	-0.03	0.01		-2.94		-2.65	0.06	0.11	0.07	0.03		18
D12S2070	8.1	-2.95	-0.42	-0.17	-0.08	-0.03		-2.54		-2.54	-0.18	-0.04	-0.01	-0.01		19
D12S395	12.7	-4.54	-0.65	-0.3	-0.15	-0.07		-3.61		-3.61	-0.26	-0.11	-0.07	-0.04		20
D12S2078	13.2	-infinity	-0.82	-0.27	-0.06	0.01		-infinity		-infinity	-1.28	-0.6	-0.28	-0.1		21
D12S1045	6	-infinity	-1.3	-0.48	-0.16	-0.03		-infinity		-infinity	-1.77	-0.84	-0.4	-0.15		22
D12S392		-infinity	-1.11	-0.35	-0.07	0.02		-7.42		-infinity	-1.39	-0.59	-0.23	-0.06		23

Table 24. Chromosome 13 two point lod score results

Marker name	Distance to next marker (cM)	MLINK analysis, affecteds and unaffecteds, V:7 classed as affected						GENEHUNTER analysis, affecteds only, V:7 classed as unknown		MLINK analysis, affecteds only, V:7 classed as affected						Multipoint graph locus number
		Recombination fraction								Recombination fraction						
		0	0.1	0.2	0.3	0.4				0	0.1	0.2	0.3	0.4		
D13S787	17.3	-2.61	-0.45	-0.26	-0.13	-0.06		-2.17		-2.16	0.29	0.37	0.27	0.12		1
D13S893	7.8	-infinity	-1.21	-0.52	-0.2	-0.05		-2.58		-2.92	-0.22	-0.06	-0.02	-0.01		2
D13S894	6.5	-infinity	-0.44	-0.19	-0.07	-0.01		0.43		0.44	0.25	0.12	0.05	0.02		3
D13S325	8.6	-infinity	-1.04	-0.42	-0.17	-0.05		-infinity		-infinity	-0.84	-0.41	-0.21	-0.09		4
D13S788	5.9	-infinity	-0.75	-0.05	0.11	0.08		-10.47		-10.17	-0.77	-0.21	-0.03	0.02		5
D13S318	6.8	-infinity	0.07	0.31	0.27	0.14		-2.80		-2.5	0.02	0.07	0.05	0.02		6
D13S800	12.1	-2.48	0.21	0.22	0.14	0.04		-3.07		-2.77	-0.03	0.05	0.04	0.02		7
GATA8G07	4.4	-2.96	-0.18	-0.07	-0.06	-0.06		-3.24		-2.94	-0.16	-0.04	-0.02	-0.02		8
D13S886	8.2	-infinity	-0.68	-0.18	-0.04	-0.02		-7.13		-6.82	-0.58	-0.15	-0.02	0		9
D13S793	7.9	-1.8	0.78	0.67	0.45	0.22		-2.15		-2.15	0.49	0.44	0.28	0.12		10
D13S779	8.9	-infinity	-0.92	-0.31	-0.08	0		-6.27		-6.27	-0.44	-0.11	-0.01	0.01		11
D13S797	7.2	-2.1	0.27	0.3	0.21	0.08		-3.13		-2.27	0.09	0.15	0.1	0.04		12
D13S895		2.82	2.22	1.61	1	0.45		1.83		2.13	1.64	1.14	0.67	0.28		13

Table 25. Chromosome 14 two point lod score results

Marker name	Distance to next marker (cM)	MLINK analysis, affecteds and unaffecteds, V:7 classed as affected						GENEHUNTER analysis, affecteds only, V:7 classed as unknown		MLINK analysis, affecteds only, V:7 classed as affected						Multipoint graph locus number
		Recombination fraction								Recombination fraction						
		0	0.1	0.2	0.3	0.4				0	0.1	0.2	0.3	0.4		
D14S742	11.1	-infinity	-0.28	0.03	0.1	0.08		-2.70		-infinity	-0.65	-0.25	-0.08	-0.02		1
D14S1280	3.6	-infinity	-0.72	-0.3	-0.11	-0.02		-2.93		-infinity	-0.77	-0.32	-0.12	-0.03		2
D14S608	5.5	-infinity	-0.3	0.01	0.07	0.05		-2.49		-infinity	-0.6	-0.22	-0.09	-0.03		3
D14S121	8.4															
D14S599	4.7	-infinity	-0.21	0.07	0.12	0.09		-2.78		-infinity	-0.7	-0.29	-0.11	-0.03		4
D14S306	3.5	-infinity	-0.31	0.01	0.09	0.07		-2.46		-infinity	-0.7	-0.29	-0.12	-0.04		5
D14S583	10.1	-infinity	-0.46	-0.11	0	0.03		-2.67		-infinity	-0.58	-0.18	-0.03	0.02		6
D14S587	8.8	-infinity	-0.26	0	0.03	0.01		-infinity		-infinity	-0.26	0	0.03	0.01		7
D14S592	6.4	-infinity	0.02	0.05	0.04	0.02		0.69		-infinity	-0.19	-0.05	-0.01	0		8
D14S588	11.7	1.07	0.83	0.59	0.37	0.18		0.72		0.72	0.53	0.35	0.2	0.08		9
D14S539	7.3															
D14S606	4.3															
D14S616	3.5	-infinity	0.47	0.55	0.41	0.21		-2.27		-infinity	-0.34	-0.06	0.01	0.02		10
D14S1279	11															
D14S617	6.3	-infinity	-1.02	-0.36	-0.09	0.01		-6.83		-infinity	-1.28	-0.56	-0.23	-0.07		11
GATA168F06	2.8	0.64	0.48	0.31	0.16	0.06		0.94		0.94	0.67	0.42	0.21	0.08		12
D14S605	5.4	-2.93	-0.14	-0.03	-0.01	0		-2.84		-2.84	-0.07	0.02	0.03	0.01		13
D14S614	4.1	-infinity	-0.81	-0.38	-0.17	-0.06		-2.84		-infinity	-0.58	-0.21	-0.07	-0.01		14

Marker name	Distance to next marker (cM)	MLINK analysis, affecteds and unaffecteds, V:7 classed as affected						GENEHUNTER analysis, affecteds only, V:7 classed as unknown		MLINK analysis, affecteds only, V:7 classed as affected						Multipoint graph locus number
		Recombination fraction								Recombination fraction						
		0	0.1	0.2	0.3	0.4				0	0.1	0.2	0.3	0.4		
D14S1426		-infinity	-1.09	-0.46	-0.19	-0.06		-6.78		-infinity	-0.65	-0.26	-0.1	-0.03		15

Table 26. Chromosome 15 two point lod score results

Marker name	Distance to next marker (cM)	MLINK analysis, affecteds and unaffecteds, V:7 classed as affected						GENEHUNTER analysis, affecteds only, V:7 classed as unknown		MLINK analysis, affecteds only, V:7 classed as affected						Multipoint graph locus number
		Recombination fraction								Recombination fraction						
		0	0.1	0.2	0.3	0.4				0	0.1	0.2	0.3	0.4		
D15S817	8.2	-infinity	-0.4	0.05	0.15	0.11		-6.87		-infinity	-0.9	-0.31	-0.08	0		1
D15S217	14.6	-infinity	-1.54	-0.73	-0.37	-0.15		-infinity		-infinity	-0.51	-0.22	-0.12	-0.05		2
D15S231	5.8	-3.08	-0.32	-0.12	-0.04	-0.01		-2.15		-2.97	-0.14	0.05	0.07	0.04		3
ACTC	2.4	-infinity	-0.37	0.19	0.28	0.18		-1.16		-infinity	0.9	0.92	0.68	0.35		4
GATA50C03	9.7	-3.33	-0.11	0.01	0.02	0.01		-2.81		-3.31	-0.16	-0.02	0.01	0.01		5
D15S659	5.2	-infinity	-1.09	-0.47	-0.2	-0.06		-2.10		-infinity	-0.41	-0.1	-0.01	0.01		6
D15S648	6.4	-infinity	-0.07	0.1	0.08	0.04		-2.38		-infinity	-0.5	-0.15	-0.03	0		7
D15S643	7	-infinity	-2.18	-0.98	-0.42	-0.13		-5.96		-infinity	-0.89	-0.31	-0.09	-0.01		8
GATA151F03	11.1	-infinity	-0.13	0.18	0.21	0.14		-2.51		-2.51	0.2	0.25	0.21	0.12		9
D15S818	8.8															
D15S655	10.5	-infinity	-1.51	-0.63	-0.26	-0.09		-infinity		-infinity	-1.43	-0.62	-0.26	-0.08		10
D15S652	8.5	-infinity	-0.18	0.05	0.07	0.04		1.27		-infinity	0.2	0.17	0.09	0.04		11
D15S816	4.9	-infinity	0.31	0.4	0.29	0.14		-3.08		-infinity	-0.32	-0.03	0.04	0.04		12
D15S657	6.3	-infinity	-2.98	-1.47	-0.72	-0.27		-infinity		-infinity	-1.31	-0.63	-0.31	-0.11		13
D15S230	20.6	-infinity	0.02	-0.03	-0.09	-0.08		-infinity		-infinity	-0.11	-0.07	-0.08	-0.06		14

Table 27. Chromosome 16 two point lod score results

Marker name	Distance to next marker (cM)	MLINK analysis, affecteds and unaffecteds, V:7 classed as affected						GENEHUNTER analysis, affecteds only, V:7 classed as unknown		MLINK analysis, affecteds only, V:7 classed as affected						Multipoint graph locus number
		Recombination fraction								Recombination fraction						
		0	0.1	0.2	0.3	0.4				0	0.1	0.2	0.3	0.4		
D16S2622	6.3	-infinity	-1.1	-0.44	-0.15	-0.03		-6.93		-6.62	-0.4	-0.09	-0.01	0		1
ATA41E04	12.1	-infinity	-0.67	-0.27	-0.11	-0.03		-3.63		-3.33	-0.38	-0.13	-0.04	-0.01		2
D16S748	8.7	-infinity	-0.26	-0.03	0.01	0.01		-2.75		-infinity	-0.68	-0.28	-0.11	-0.03		3
D16S764	9.2	-1.15	0.28	0.39	0.28	0.13		-1.29		-1.28	0.94	0.78	0.51	0.24		4
D16S749	11.2	-infinity	0.3	0.27	0.16	0.06		1.34		1.34	1	0.67	0.38	0.16		5
D16S769	7.9	-2.67	-0.23	-0.05	0	0		-2.67		-2.67	-0.23	-0.05	0	0		6
D16S753	5.8															
ATA55A11	2	-infinity	-1.03	-0.41	-0.15	-0.03		-6.71		-infinity	-0.81	-0.32	-0.12	-0.03		7
D16S757	6.4	-infinity	-1.16	-0.46	-0.15	-0.02		-2.66		-infinity	-0.31	-0.03	0.03	0.03		8
D16S3253	11.2	-infinity	0.09	0.16	0.12	0.06		0.89		-infinity	0.16	0.16	0.09	0.04		9
GATA67G11	6	-infinity	-0.96	-0.33	-0.1	-0.01		-6.90		-infinity	-0.52	-0.18	-0.07	-0.02		10
D16S2624	16.2	-infinity	-0.06	0.1	0.12	0.07		0.18		-infinity	-0.16	0.05	0.06	0.03		11
D16S750	28.2	-0.4	-0.3	-0.17	-0.07	-0.01		-0.23		-0.22	-0.13	-0.06	-0.02	-0.01		12
D16S539	5.3	-infinity	-1.43	-0.65	-0.29	-0.11		-5.59		-7.17	-0.23	0.07	0.11	0.07		13
D16S2621		-3.75	-0.8	-0.33	-0.13	-0.04		-2.78		-2.78	-0.11	-0.01	0.01	0.01		14

Table 28. Chromosome 17 two point lod score results

Marker name	Distance to next marker (cM)	MLINK analysis, affecteds and unaffecteds, V:7 classed as affected						GENEHUNTER analysis, affecteds only, V:7 classed as unknown		MLINK analysis, affecteds only, V:7 classed as affected						Multipoint graph locus number
		Recombination fraction								Recombination fraction						
		0	0.1	0.2	0.3	0.4				0	0.1	0.2	0.3	0.4		
D17S1308	11.6	-infinity	-1.1	-0.48	-0.18	-0.04		-3.05		-6.59	-0.52	-0.13	0	0.03		1
D17D1298	9.3	-3.12	-0.51	-0.29	-0.17	-0.08		-2.75		-2.75	-0.2	-0.06	-0.02	-0.01		2
D17S1537	10.2	-2.23	0.5	0.49	0.34	0.16		-2.39		-2.08	0.54	0.48	0.32	0.15		3
D17S974	4.5															
D17S969	10.5	-3.04	-0.39	-0.15	-0.05	-0.01		-2.72		-2.71	-0.09	0.01	0.03	0.02		4
D17S900	9															
GATA185H04	6.4	-5.24	0.43	0.52	0.37	0.17		-6.69		-6.38	-0.32	-0.04	0.01	0		5
D17S1294	5.6	-3.5	-0.52	-0.04	0.08	0.07		-3.46		-3.46	-0.51	-0.11	0.01	0.03		6
D17S1293	6.6															
D17S1299	4.5	-infinity	0.24	0.28	0.22	0.12		-3.19		-3.19	-0.05	0.05	0.05	0.03		7
D17S2180	17.1															
D17S1290	9.9															
ATA43A10	8.6															
D17S1534	9.2															
D17S968		-infinity	-0.68	-0.1	0.09	0.1		-2.60		-infinity	-0.47	-0.13	-0.02	0.01		8

Table 29. Chromosome 18 two point lod score results

Marker name	Distance to next marker (cM)	MLINK analysis, affecteds and unaffecteds, V:7 classed as affected						GENEHUNTER analysis, affecteds only, V:7 classed as unknown		MLINK analysis, affecteds only, V:7 classed as affected						Multipoint graph locus number
		Recombination fraction								Recombination fraction						
		0	0.1	0.2	0.3	0.4				0	0.1	0.2	0.3	0.4		
GATA178F11	9.5	-infinity	0.05	0.41	0.39	0.22		-3.21		-2.91	-0.36	-0.05	0.12	0.07		1
D18S853	2.5	-3.04	-0.35	-0.16	-0.09	-0.04		-3.01		-3.01	-0.3	-0.11	-0.05	-0.02		2
D18S976	6.5	-infinity	-1.21	-0.51	-0.21	-0.07		-6.57		-infinity	-0.94	-0.36	-0.14	-0.04		3
D18S973	11.4	-infinity	0.51	0.42	0.25	0.1		-infinity		-infinity	0.25	0.22	0.1	0.02		4
D18S843	11.2	-infinity	-0.57	-0.28	-0.16	-0.07		-2.55		-infinity	-0.45	-0.14	-0.04	-0.01		5
D18S542	16.1															
D18S877	2.9	0.73	0.58	0.42	0.28	0.14		-1.20		0.26	0.18	0.11	0.05	0.02		6
D18S847	4.1	-2.21	-0.13	0.14	0.17	0.11		-2.34		-2.34	-0.23	0.06	0.12	0.08		7
D18S974	2.1	-infinity	-0.84	-0.12	0.09	0.1		-infinity		-infinity	-1.3	-0.47	-0.15	-0.02		8
D18S978	2.7	-infinity	-0.85	-0.51	-0.3	-0.13		-infinity		-infinity	-0.81	-0.41	-0.22	-0.09		9
D18S872	9.8	-infinity	-1.13	-0.47	-0.19	-0.06		0.74		-infinity	-0.18	-0.04	0	0.01		10
D18S851	3.9	-infinity	0.61	0.52	0.36	0.19		-2.84		-infinity	0.03	0.1	0.08	0.04		11
D18S858	9.7	-infinity	-0.07	0.17	0.18	0.1		-2.49		-2.19	0.2	0.22	0.15	0.07		12
D18S1357	9.4	-infinity	-0.44	-0.11	0	0.02		-2.43		-infinity	-0.26	0	0.05	0.03		13
GATA26C03	1.9	-infinity	-0.02	0.39	0.4	0.24		-1.55		-infinity	0.43	0.55	0.42	0.21		14
D18S969	2.5	-infinity	-0.51	-0.17	-0.07	-0.03		-infinity		-infinity	-0.76	-0.37	-0.21	-0.11		15
D18S857	8.8	-infinity	-0.44	-0.12	-0.02	0.01		-infinity		-infinity	-0.92	-0.47	-0.25	-0.1		16
D18S541	7.9	-2.26	0.18	0.23	0.17	0.08		-2.00		-2.02	0.35	0.36	0.25	0.11		17

Marker name	Distance to next marker (cM)	MLINK analysis, affecteds and unaffecteds, V:7 classed as affected						GENEHUNTER analysis, affecteds only, V:7 classed as unknown		MLINK analysis, affecteds only, V:7 classed as affected						Multipoint graph locus number
		Recombination fraction								Recombination fraction						
		0	0.1	0.2	0.3	0.4				0	0.1	0.2	0.3	0.4		
GATA177C03	3.2	-2.52	-0.02	0.08	0.07	0.03		-2.53		-2.53	-0.05	0.04	0.04	0.02		18
D18S844		-infinity	-0.87	-0.3	-0.08	0		-infinity		-infinity	-1.25	-0.65	-0.35	-0.15		19

Table 30. Chromosome 19 two point lod score results

Marker name	Distance to next marker (cM)	MLINK analysis, affecteds and unaffecteds, V:7 classed as affected						GENEHUNTER analysis, affecteds only, V:7 classed as unknown		MLINK analysis, affecteds only, V:7 classed as affected						Multipoint graph locus number
		Recombination fraction								Recombination fraction						
		0	0.1	0.2	0.3	0.4				0	0.1	0.2	0.3	0.4		
D19S591	11.7	-infinity	-0.96	-0.52	-0.29	-0.13		0.53		0.74	0.46	0.24	0.1	0.03		1
D19S1034	3.5	-0.314	-0.29	0.03	0.1	0.07		-2.57		-2.57	-0.03	0.1	0.1	0.06		2
D19S592	9.1	-infinity	-1.55	-0.8	-0.42	-0.17		-infinity		-infinity	-0.88	-0.45	-0.24	-0.11		3
D19S586	4.1	-infinity	-0.81	-0.34	-0.13	-0.03		-2.96		-3.27	-0.49	-0.15	-0.04	0		4
GATA134B01	6.3	-2.76	-0.33	-0.14	-0.07	-0.03		-2.72		-2.71	-0.29	-0.1	-0.04	-0.02		5
D19S714	3.8	-infinity	-0.87	-0.31	-0.1	-0.01		-infinity		-infinity	-0.46	-0.18	-0.1	-0.05		6
D19S593	5.8	-infinity	-1.02	-0.24	0.03	0.08		-6.94		-6.64	-0.63	-0.25	-0.11	-0.04		7
D19S1037	5.4	-infinity	-0.94	-0.36	-0.14	-0.04		0.73		-2.79	-0.12	0	0	-0.01		8
D19S433	3.8	-infinity	-0.82	-0.35	-0.14	-0.04		-0.01		-3.3	-0.42	-0.16	-0.06	-0.02		9
D19S431	2.5	-infinity	-0.81	-0.29	-0.1	-0.03		-3.69		-3.69	-0.72	-0.31	-0.14	-0.05		10
D19S719	1.9	-infinity	-0.96	-0.49	-0.24	-0.09		-3.17		-2.86	-0.09	0.01	0.01	0		11
D19S587	5.5	-2.21	0.11	0.2	0.16	0.09		-2.63		-2.63	-0.25	-0.09	-0.04	-0.02		12
GATA84G04	11.4	-infinity	-0.43	0.09	0.21	0.16		-2.52		-2.22	0.39	0.35	0.22	0.1		13
D19S541	8															
D19S585	4.6															
D19S601	3.8	-infinity	-0.73	-0.12	0.09	0.11		-2.67		-2.37	0.13	0.16	0.11	0.05		14
D19S589		-infinity	-1.36	-0.54	-0.22	-0.07		-2.98		-3.77	-0.46	-0.16	-0.08	-0.05		15

Table 31. Chromosome 20 two point lod score results

Marker name	Distance to next marker (cM)	MLINK analysis, affecteds and unaffecteds, V:7 classed as affected						GENEHUNTER analysis, affecteds only, V:7 classed as unknown		MLINK analysis, affecteds only, V:7 classed as affected						Multipoint graph locus number
		Recombination fraction								Recombination fraction						
		0	0.1	0.2	0.3	0.4				0	0.1	0.2	0.3	0.4		
D20S473	4	0.98	0.53	0.15	-0.07	-0.09		1.24		1.08	0.74	0.45	0.23	0.09		1
D20S482	5.9	0.79	0.32	-0.09	-0.3	-0.22		0.15		0.45	0.15	-0.03	-0.08	-0.06		2
D20S603	9.2	-infinity	-2.35	-1.25	-0.73	-0.36		-infinity		-infinity	-1.44	-0.78	-0.45	-0.21		3
D20S162	10.2	-infinity	-1.17	-0.59	-0.3	-0.12		-2.36		-2.36	-0.04	0.05	0.05	0.02		4
D20S604	6.6	-3.92	-0.11	0.01	0	-0.03		-3.51		-3.21	-0.04	0.07	0.06	0.02		5
D20S470	3.3	-infinity	-0.3	-0.01	0.05	0.04		-2.44		-2.53	0.02	0.14	0.13	0.07		6
D20S471	5.1	-infinity	1.08	1.04	0.72	0.34		-infinity		-infinity	1.23	1	0.63	0.26		7
D20S477	4.3	-infinity	-0.23	0.13	0.18	0.12		-infinity		-infinity	-0.2	0.06	0.09	0.05		8
GATA65E01	2.2	-6.27	-0.27	0.05	0.08	0.03		-6.45		-6.15	-0.1	0.12	0.12	0.06		9
D20S478	2	-11.75	-1.53	-0.68	-0.31	-0.12		-10.64		-10.64	-0.95	-0.34	-0.1	-0.01		10
D20S607	7.8	-infinity	0.24	0.42	0.35	0.19		-infinity		-infinity	-0.52	-0.18	-0.08	-0.03		11
D20S481	3.7	-infinity	0.16	0.34	0.26	0.12		-infinity		-infinity	-0.24	-0.01	0.01	-0.01		12
D20S159	11.7	-infinity	-0.97	-0.37	-0.13	-0.04		-infinity		-infinity	-1.44	-0.71	-0.35	-0.13		13
D20S480	2	-infinity	-0.15	0.14	0.15	0.08		-6.99		-6.69	-0.32	0	0.06	0.04		14
D20S1085	3.7	-6.59	-0.26	0.05	0.1	0.05		-6.77		-6.46	-0.34	-0.03	0.03	0.02		15
D20S1082		-infinity	-0.5	-0.16	-0.04	0		-6.99		-6.99	-0.44	-0.11	-0.01	0.01		16

Table 32. Chromosome 21 two point lod score results

Marker name	Distance to next marker (cM)	MLINK analysis, affecteds and unaffecteds, V:7 classed as affected						GENEHUNTER analysis, affecteds only, V:7 classed as unknown		MLINK analysis, affecteds only, V:7 classed as affected						Multipoint graph locus number
		Recombination fraction								Recombination fraction						
		0	0.1	0.2	0.3	0.4				0	0.1	0.2	0.3	0.4		
D21S1432	6.1															
D21S1433	6.8															
D21S1438	6.4	0.63	0.47	0.34	0.22	0.12		0.17		0.17	0.09	0.04	0.01	0		1
D21S1435	4.2	-infinity	0.14	0.32	0.28	0.16		-2.77		-7.16	-0.59	-0.22	-0.08	-0.02		2
D21S2052	1.3	-2.45	-0.21	-0.13	-0.1	-0.06		-2.76		-2.76	-0.23	-0.04	0.01	0		3
D21S1270	15.4															
D21S1439	2.5	-infinity	-0.59	-0.21	-0.07	-0.02		-2.27		-2.27	0.21	0.21	0.13	0.06		4
GATA188F04	19	-infinity	-1.98	-0.9	-0.41	-0.15		-infinity		-infinity	-1.83	-0.93	-0.49	-0.21		5
D21S1446		-infinity	-0.53	-0.05	0.06	0.05		-infinity		-infinity	-0.11	0.12	0.12	0.05		6

Table 33. Chromosome 22 two point lod score results

Marker name	Distance to next marker (cM)	MLINK analysis, affecteds and unaffecteds, V:7 classed as affected						GENEHUNTER analysis, affecteds only, V:7 classed as unknown		MLINK analysis, affecteds only, V:7 classed as affected						Multipoint graph locus number
		Recombination fraction								Recombination fraction						
		0	0.1	0.2	0.3	0.4				0	0.1	0.2	0.3	0.4		
D22S1266	13.3	0.59	0.4	0.24	0.12	0.04		0.77		0.77	0.54	0.34	0.18	0.07		1
D22S686	4	-infinity	-0.96	-0.37	-0.13	-0.03		-6.57		-6.27	-0.38	-0.06	0.02	0.02		2
D22S1685	6.8															
D22S690	7.2	-11.4	-1.64	-0.8	-0.38	-0.14		-10.62		-10.62	-1.11	-0.45	-0.17	-0.04		3
D22S689	4.9	-infinity	-1.32	-0.63	-0.33	-0.14		-infinity		-infinity	-0.37	-0.11	-0.06	-0.04		4
D22S691	3.5	-infinity	-1.16	-0.48	-0.19	-0.05		-7.29		-6.99	-0.65	-0.19	-0.05	-0.01		5
D22S683	3.8															
D22S692	2.6	-infinity	-1.51	-0.62	-0.23	-0.05		-infinity		-infinity	-1.09	-0.48	-0.22	-0.08		6
D22S445	13.8	-infinity	-0.39	-0.17	-0.06	0		0.28		0.58	0.39	0.24	0.13	0.05		7
D22S1267		-2.87	-0.22	-0.06	-0.02	-0.01		-2.51		-2.51	0.02	0.09	0.07	0.03		8

5.2.2 Two point lod scores of interest

Chromosome 5q34-5q35

The highest two point lod score obtained from the genome scan was 3.18 ($\theta = 0$, GENEHUNTER analysis) for marker D5S1471. A nearby marker D5S1713 yielded a two point lod score of 2.63 ($\theta = 0$, GENEHUNTER analysis), and these results were highly suggestive of linkage to this region. However, examination of the haplotypes for these two markers and the intervening marker D5S1456, shown in figure 9, reveals that all the affected individuals share a common allele for each of these markers, but also that these haplotypes are not identical by descent. All the affected individuals share allele 3 for marker D5S1471, resulting in the high two point lod score, but this allele is also present in V:7 and the unaffected individual V:6. All the affected individuals also possess a common allele for marker D5S1713, but once again this allele is also shared by individuals V:6 and V:7. Although the affected individual V:5 has inherited allele 3 for marker D5S1471 and allele 1 for marker D5S1713, both of which are shared by all the other affected individuals, inspection of the haplotypes for the intervening marker D5S1456 shows us that he must have inherited allele 5, which the other affected individuals also possess, from his unaffected mother rather than from his affected father. Provided that individual V:5 is not a phenocopy the haplotypes suggest that linkage to this region is unlikely since this would require a double recombination to have occurred around marker D5S1456.

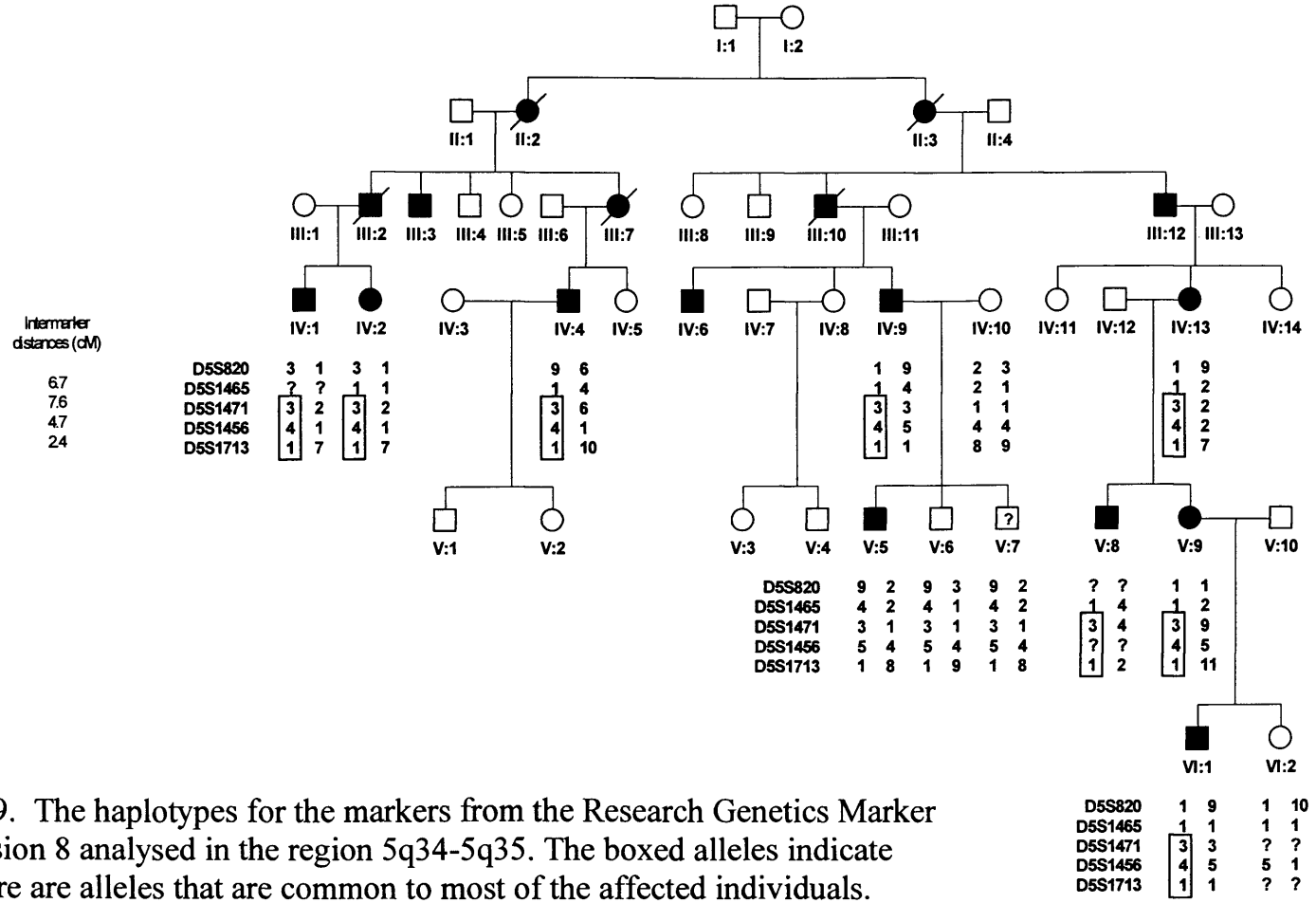


Figure 9. The haplotypes for the markers from the Research Genetics Marker Set version 8 analysed in the region 5q34-5q35. The boxed alleles indicate that there are alleles that are common to most of the affected individuals. However, the affected individual V:5 must have inherited allele 4 for marker D5S1456 from his unaffected mother, not from his affected father.

One marker, D5S1495, 35 centimorgans centromeric to marker D5S1471 yielded a lod score of 1.56 ($\theta = 0$, MLINK analysis, affecteds only) on the initial analysis with individual V:7 classed as affected. On the subsequent analysis with this person classed as unknown with regards to affection status, the two point lod score fell to 1.26 ($\theta = 0$, GENEHUNTER analysis). The haplotypes around this marker are shown in figure 10, and it can be seen that all individuals, both affecteds and unaffecteds, share allele 2 for marker D5S1495.

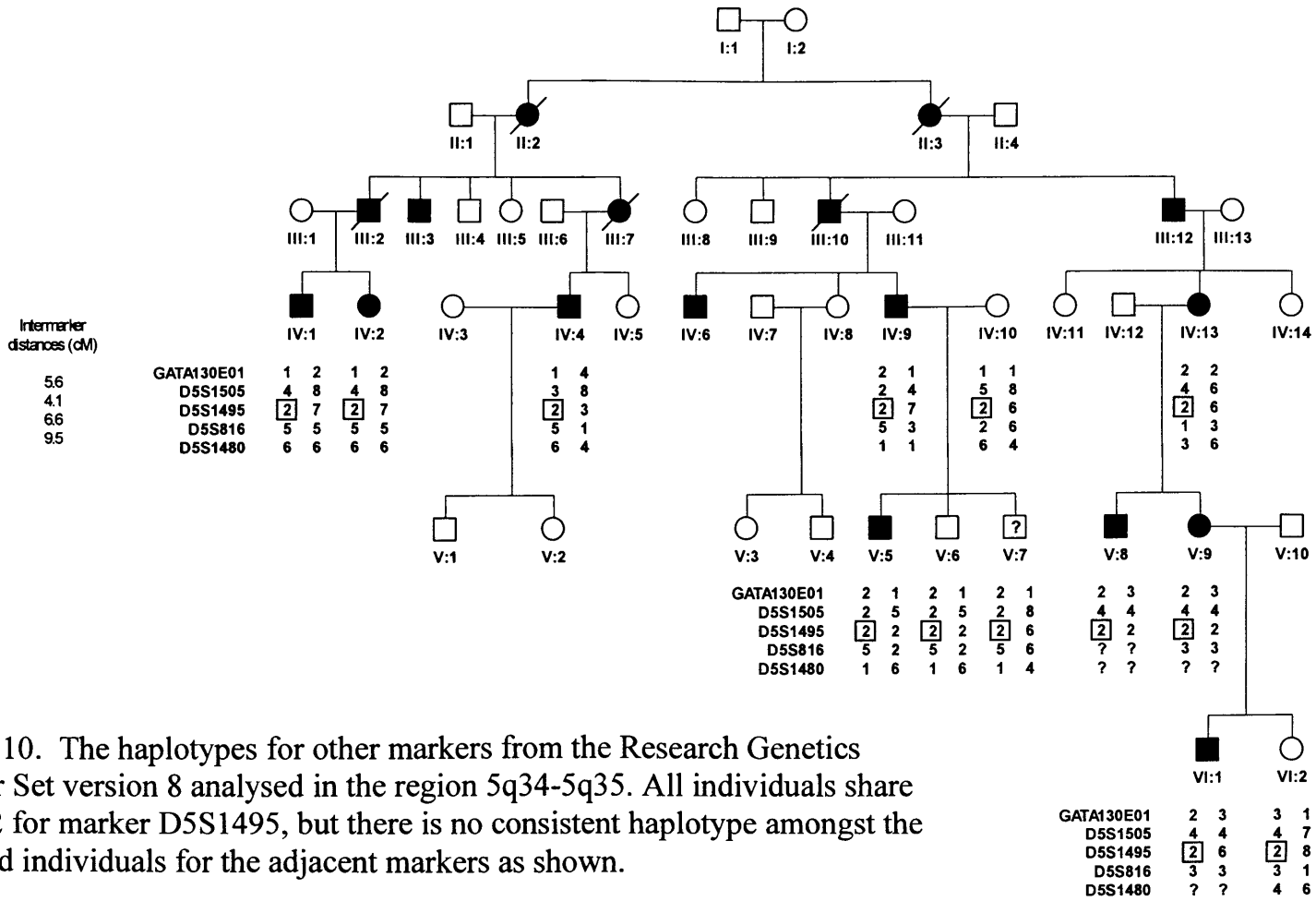


Figure 10. The haplotypes for other markers from the Research Genetics Marker Set version 8 analysed in the region 5q34-5q35. All individuals share allele 2 for marker D5S1495, but there is no consistent haplotype amongst the affected individuals for the adjacent markers as shown.

Chromosome 3q25

A two point lod score of 2.34 ($\theta = 0.1$) was generated for marker D3S4531 using the MLINK software with all individuals and with individual V:7 classified as affected, and of 1.53 ($\theta = 0.1$) using affecteds only. Analysis with individual V:7 classed as affection unknown using the GENEHUNTER software produced a two point lod score of 2.86 ($\theta = 0$). The allele nominated as 5 is common to all affected individuals, but this appears to be a common allele and is also shared by the unaffected individuals IV:10 and VI:2 (see figure 11). None of the adjacent markers show a particular allele that is common to all the affected individuals.

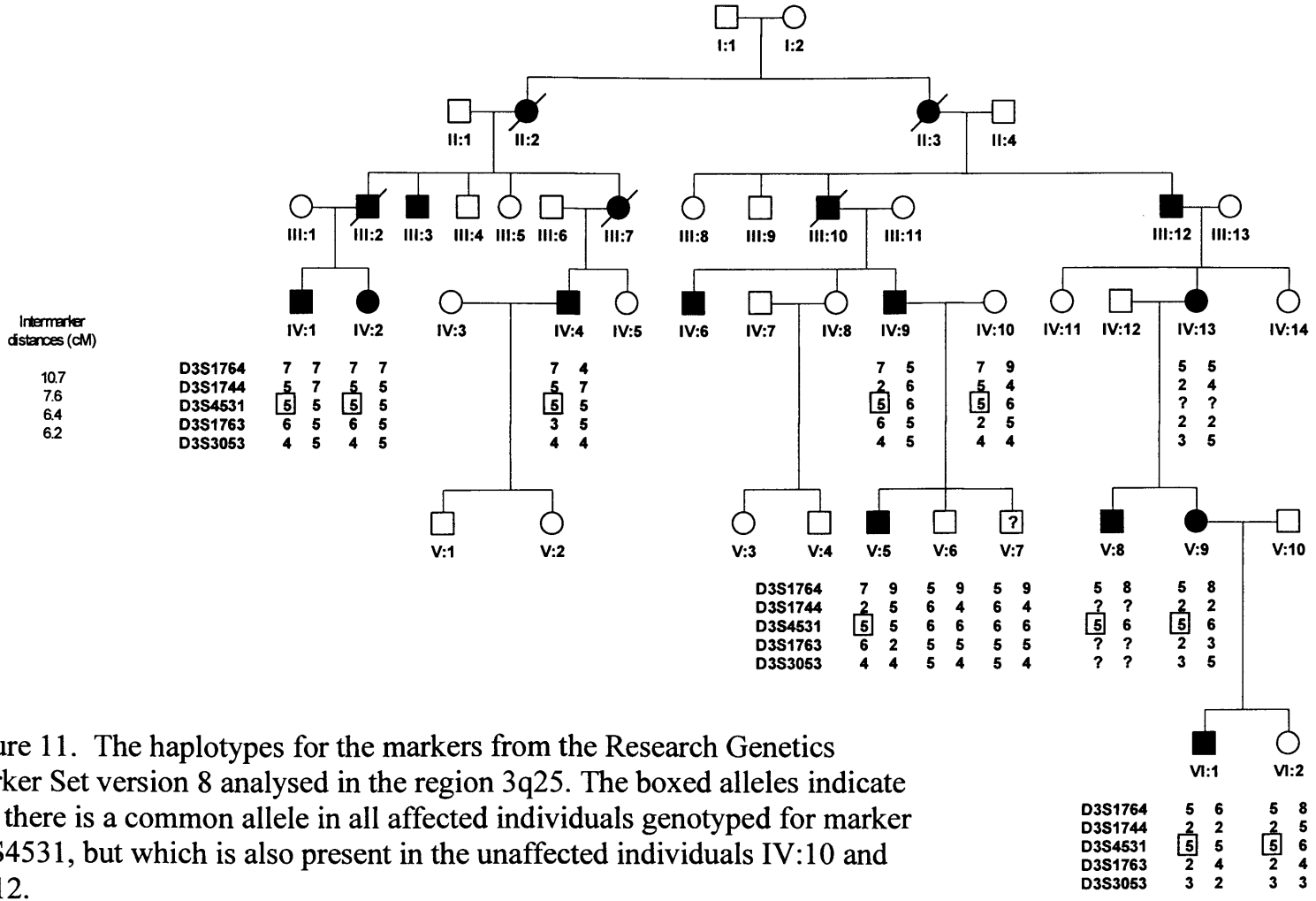


Figure 11. The haplotypes for the markers from the Research Genetics Marker Set version 8 analysed in the region 3q25. The boxed alleles indicate that there is a common allele in all affected individuals genotyped for marker D3S4531, but which is also present in the unaffected individuals IV:10 and VI:12.

Chromosome 5p14

A two point lod score of 2.17 ($\theta = 0$) was achieved for the marker D5S1470 when the data for the affected individuals alone (including individual V:7 as affected) were analysed using the MLINK software, and a score of 1.87 ($\theta = 0$) was calculated for this marker with the GENEHUNTER analysis of affecteds only with V:7 affection status unknown. The adjacent marker, GATA145D09, yielded a two point lod score of –1.29 (GENEHUNTER analysis, $\theta = 0$), although examination of the haplotype reveals that all affected individuals share a common allele for this marker as well as for D5S1470 (figure 12). The common haplotype for these two markers is also present in individual V:7 whose affection status has been questioned.

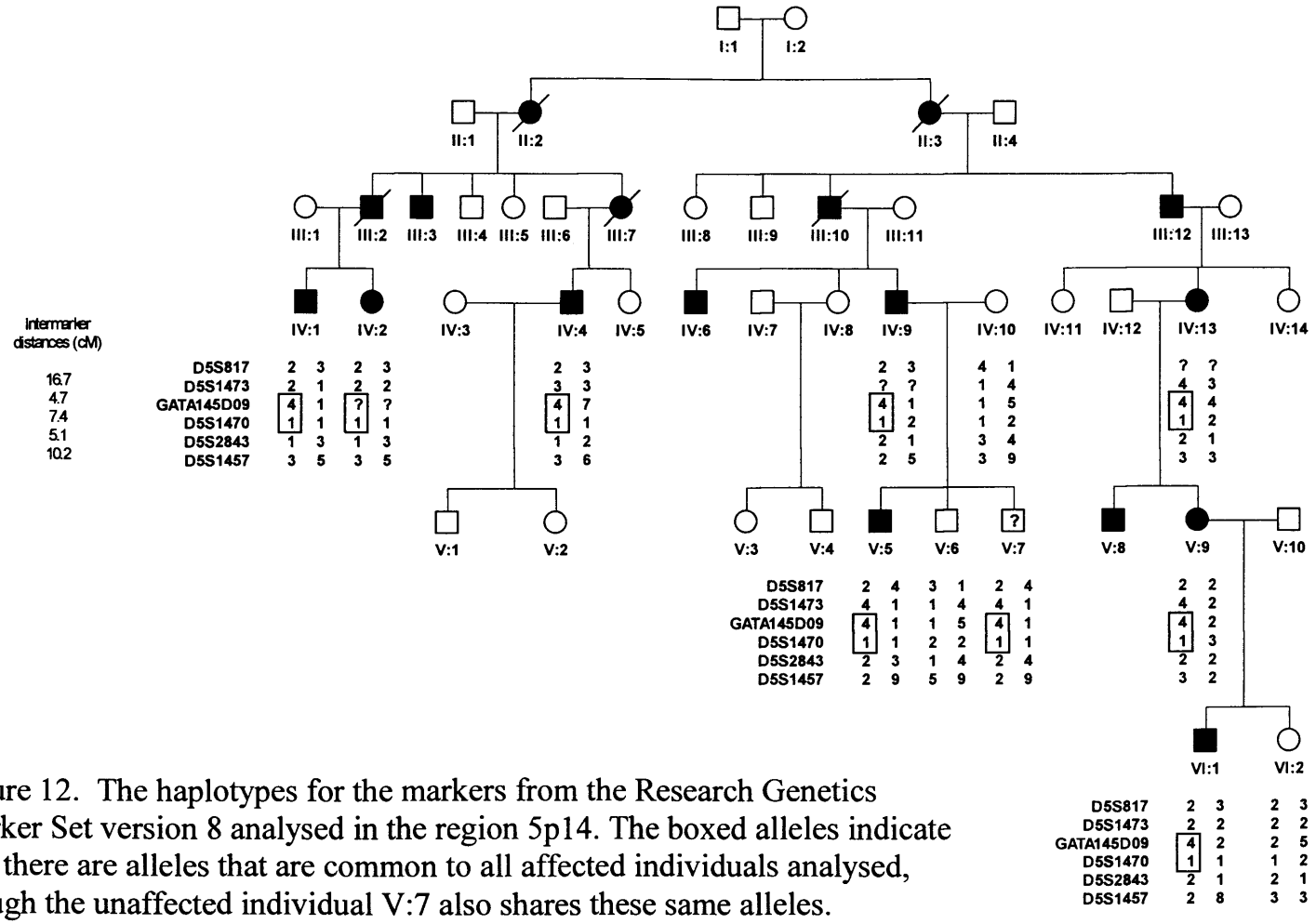


Figure 12. The haplotypes for the markers from the Research Genetics Marker Set version 8 analysed in the region 5p14. The boxed alleles indicate that there are alleles that are common to all affected individuals analysed, though the unaffected individual V:7 also shares these same alleles.

Chromosome 11q25

Marker D11S2359 produced a lod score of 1.87 ($\theta = 0$, GENEHUNTER analysis). Only ten subjects were successfully genotyped for this marker and all of these, both affected and unaffected, shared a common allele. This marker is located at the end of the long arm of chromosome 11, and the adjacent markers do not show a consistent pattern amongst the affected individuals (figure 13).

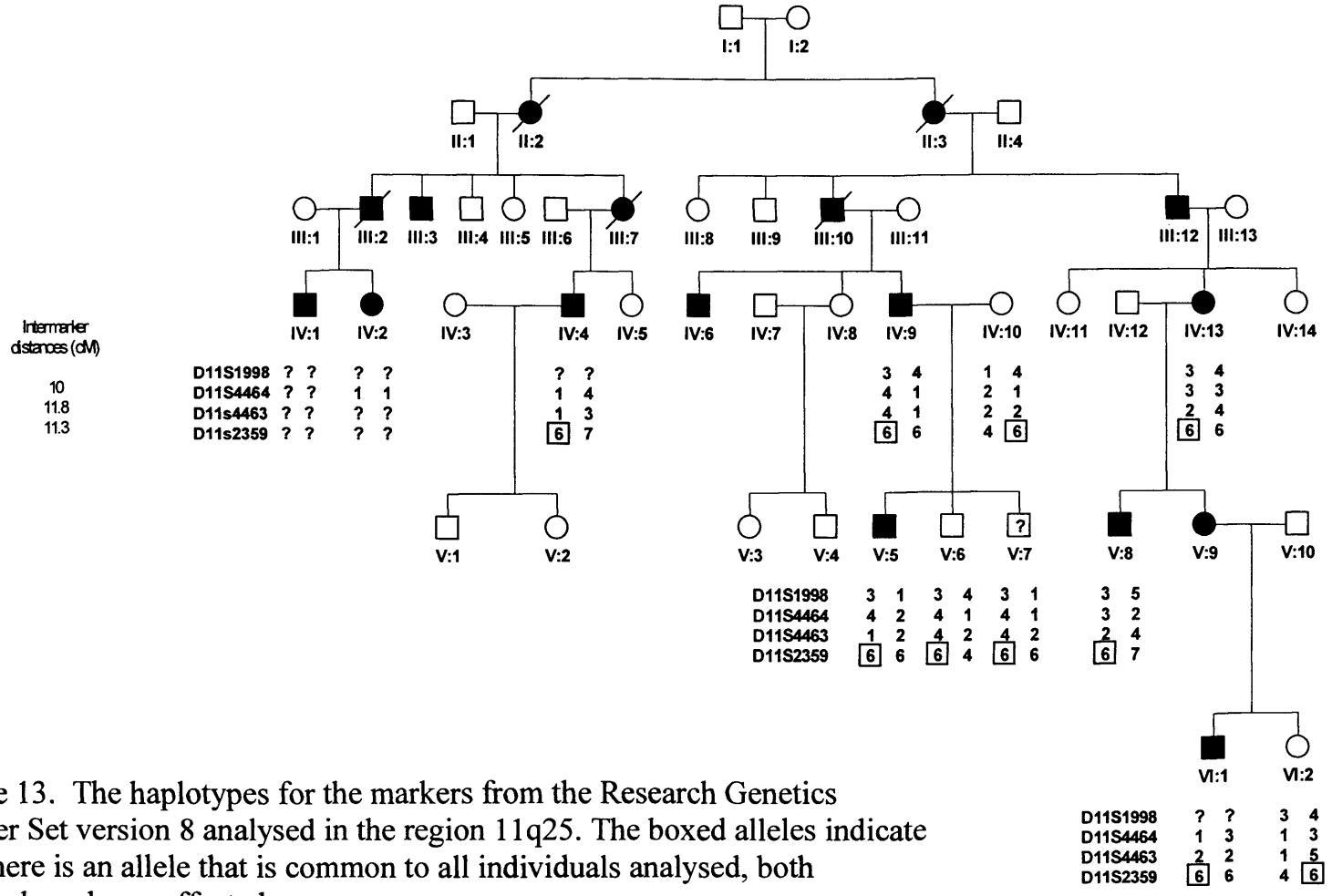


Figure 13. The haplotypes for the markers from the Research Genetics Marker Set version 8 analysed in the region 11q25. The boxed alleles indicate that there is an allele that is common to all individuals analysed, both affecteds and non-affecteds.

Chromosome 13q34

Marker D13S895 yielded a maximum two point lod score of 2.82 (LINKAGE analysis, $\theta = 0$) when both affecteds and unaffecteds were included, and a maximum two point lod score for affecteds only of 1.83 ($\theta = 0$, GENEHUNTER analysis). Linkage to adjacent markers in this region was excluded by two point lod scores less than –2 ($\theta = 0$, GENEHUNTER analysis). Analysis of the haplotypes revealed that all the affected individuals, as well as individual V:7, share allele 2 for marker D13S895, but there are no other common alleles in this region (figure 14).

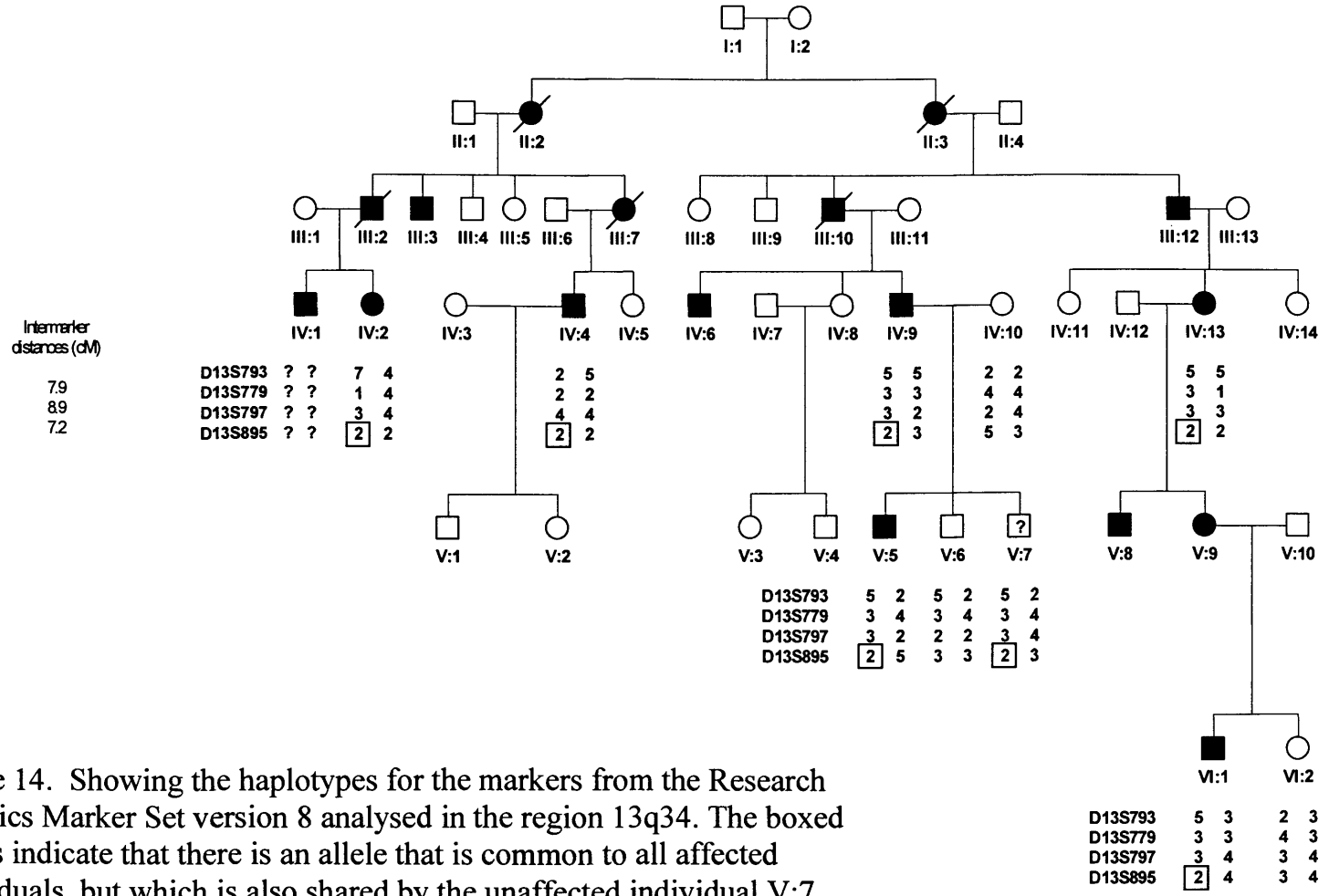


Figure 14. Showing the haplotypes for the markers from the Research Genetics Marker Set version 8 analysed in the region 13q34. The boxed alleles indicate that there is an allele that is common to all affected individuals, but which is also shared by the unaffected individual V:7.

Chromosome 2q32-2q37

Five markers in this 55.5cM region yielded positive two point lod scores; D2S1370 achieved the highest two point lod score of 1.67 ($\theta = 0$, GENEHUNTER analysis), and the adjacent marker D2S427 produced a two point lod score of 1.13 ($\theta = 0$, GENEHUNTER analysis). Two markers, D2S1384 and D1S1649, located 14.8cM centromeric to D2S1370 also yielded positive two point lod scores of 1.66 and 1.22 respectively ($\theta = 0$, GENEHUNTER analysis). A particular allele for each of these markers was found to be present in all of the affected individuals studied, but these alleles were also present in the phenotypically normal individual VI:2 (figure 15). This region was of particular interest because a separate family with an autosomal recessive phenotype {Brady, Winter, et al 2002 81 /id} showed potential linkage to this region using homozygosity mapping (personal communication M. Bitner-Glindzicz and J.Tyson)

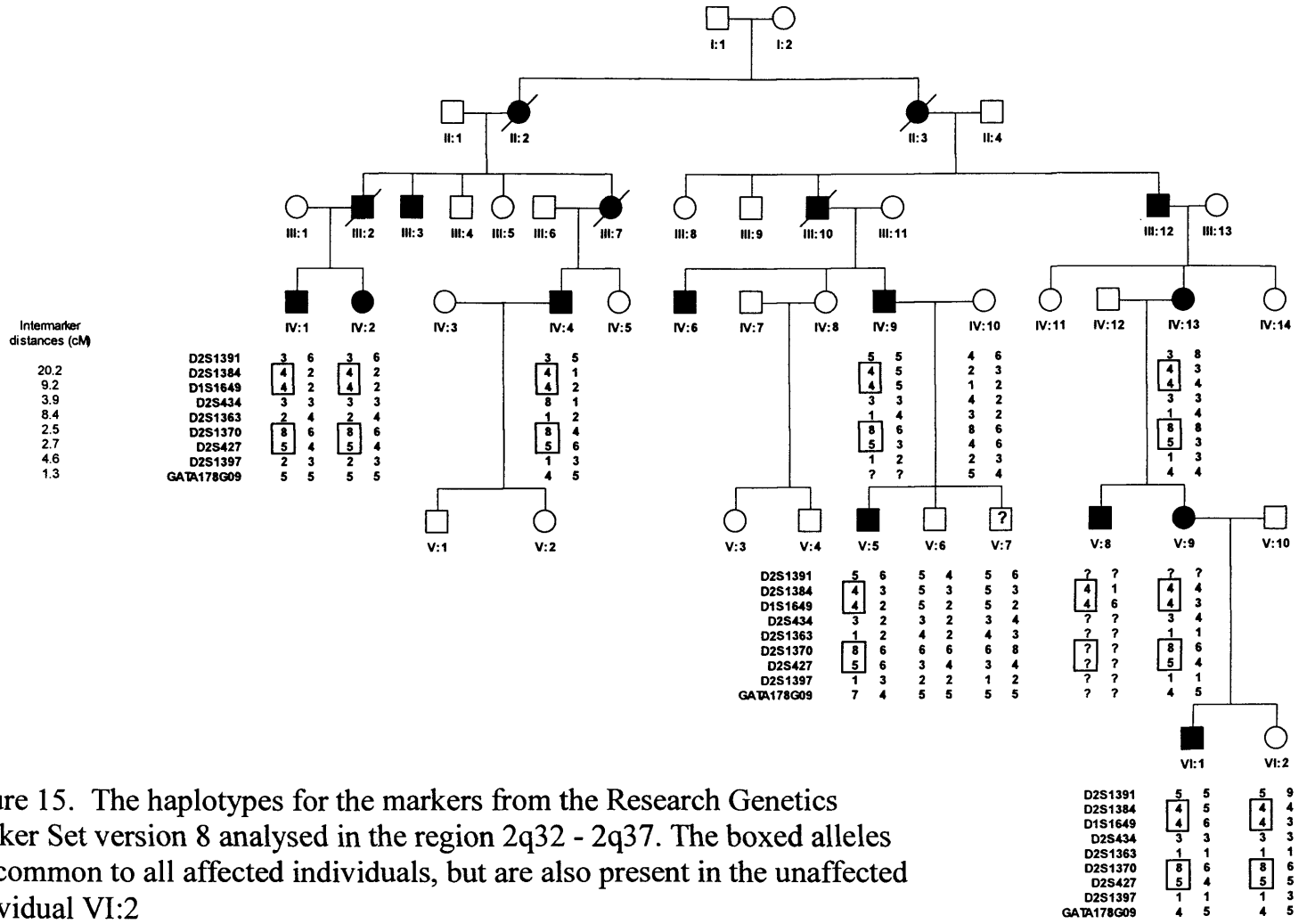


Figure 15. The haplotypes for the markers from the Research Genetics Marker Set version 8 analysed in the region 2q32 - 2q37. The boxed alleles are common to all affected individuals, but are also present in the unaffected individual VI:2

Chromosome 8q24

A two point lod score of 1.68 ($\theta = 0$, GENEHUNTER analysis) was generated from the genotyping data for marker D8S1985. All the affected individuals share a common allele for this marker and for the final marker on this chromosome, D8S1100 (figure 16). The intervening marker, D8S1462, yielded a two point lod score of –2.86. The shared alleles are also observed in the unaffected individual VI:2.

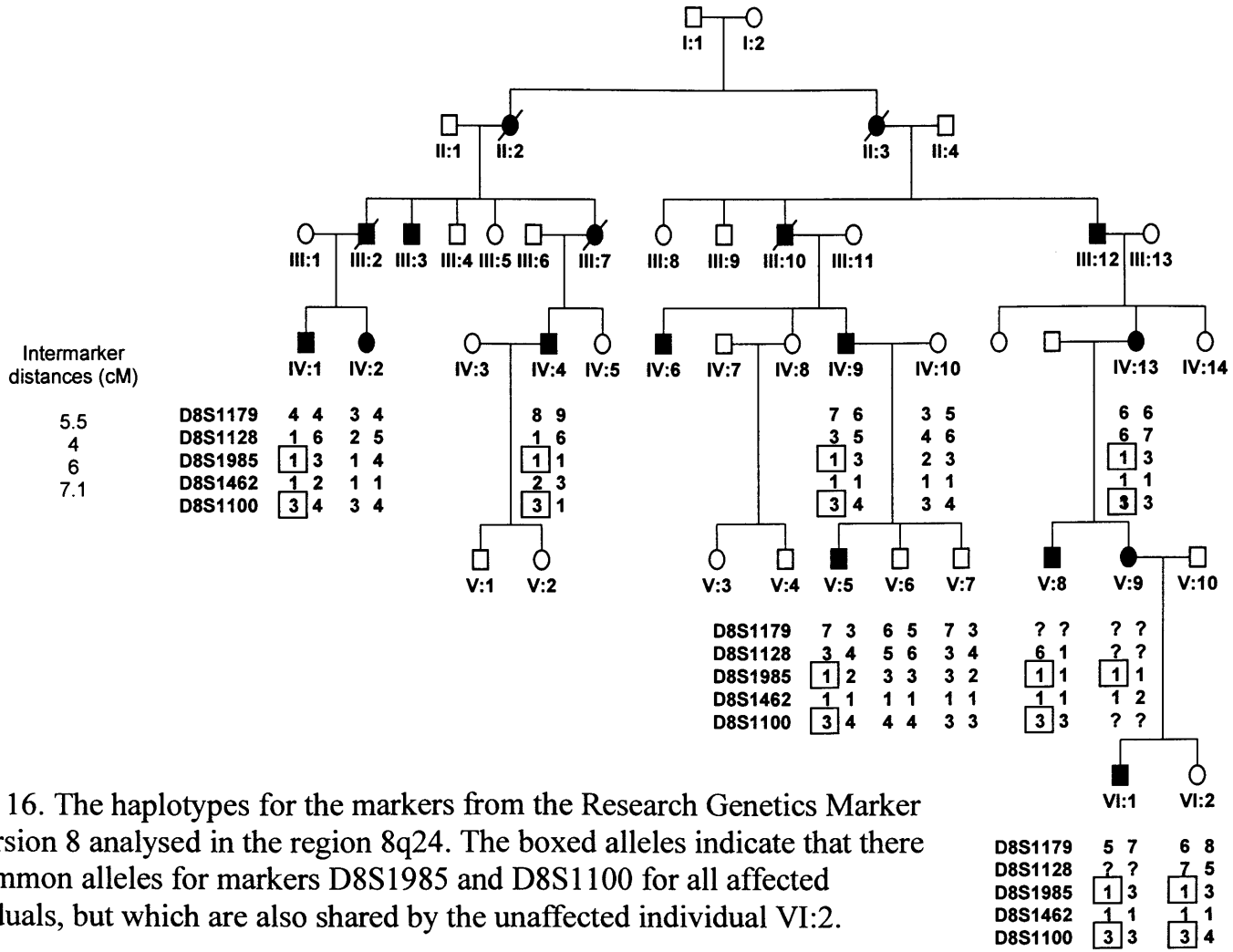


Figure 16. The haplotypes for the markers from the Research Genetics Marker Set version 8 analysed in the region 8q24. The boxed alleles indicate that there are common alleles for markers D8S1985 and D8S1100 for all affected individuals, but which are also shared by the unaffected individual VI:2.

Chromosome 16p12

Marker D16S749 yielded a maximum lod score of 1.34 ($\theta = 0$) in the second analysis using LINKAGE software (affecteds only, V:7 classed as affected), and an identical score was produced when the analysis was reperformed using the GENEHUNTER program and with the alteration made to individual V:7's affection status.

All the genotyped individuals share a common allele for both marker D16S749 and the adjacent marker D16S764, including two unaffected people and individual V:7 (figure 17). Also, individuals IV:9 and IV:10 are heterozygous for the same alleles for marker D16S764, and are identically homozygous for marker D16S749, so that the meioses that produced their children are not informative.

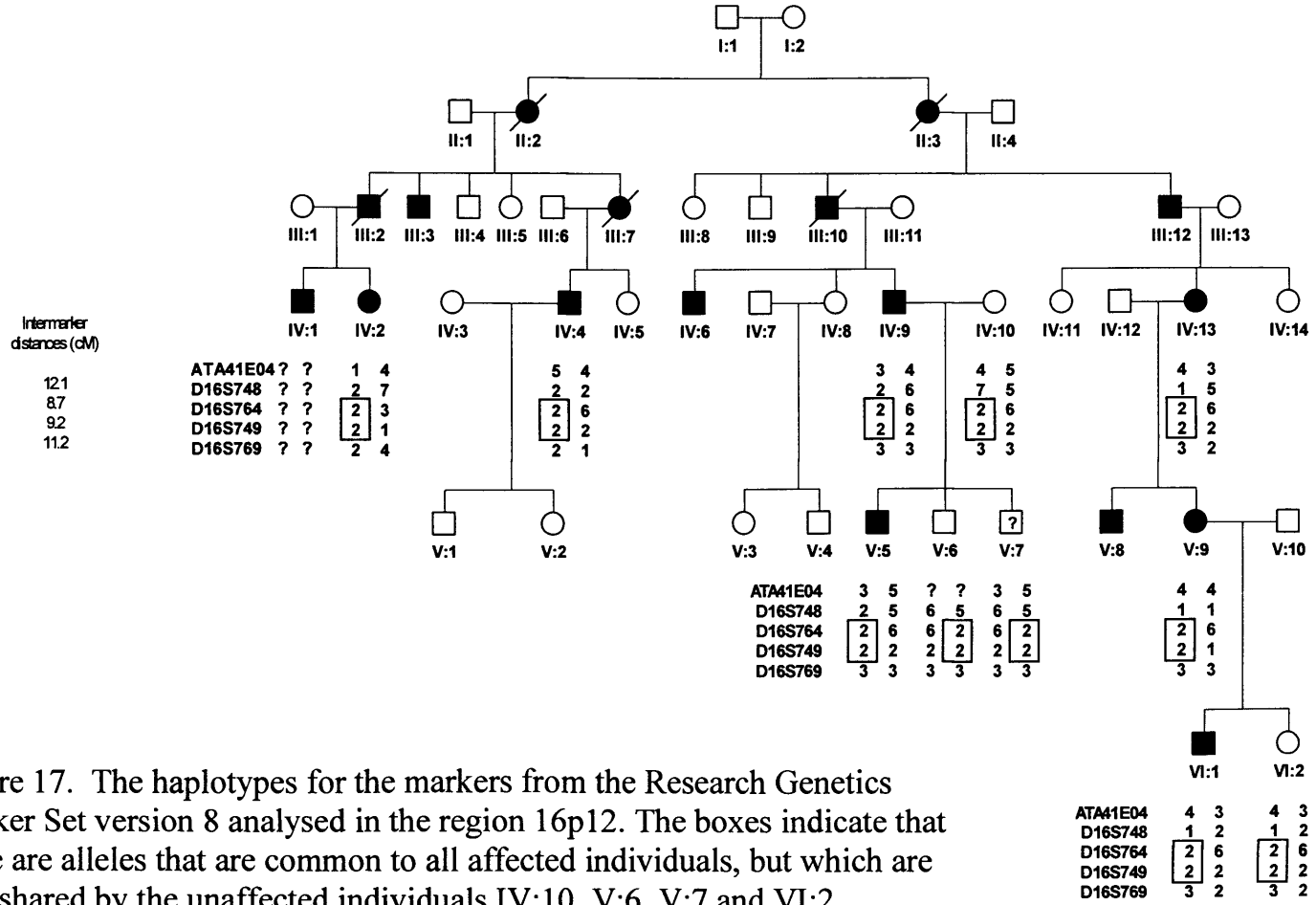


Figure 17. The haplotypes for the markers from the Research Genetics Marker Set version 8 analysed in the region 16p12. The boxes indicate that there are alleles that are common to all affected individuals, but which are also shared by the unaffected individuals IV:10, V:6, V:7 and VI:2.

Chromosome 3p25

The MLINK analysis of affecteds only, with V:7 classed as affected, produced a two point lod score of 1.89 at a recombination fraction of 0.1 for marker D3S2403 (table 14). At a recombination fraction of zero, all of the two point lod scores for this marker excluded linkage. There are no alleles that are common to all of the affected individuals in this region as shown in figure 18.

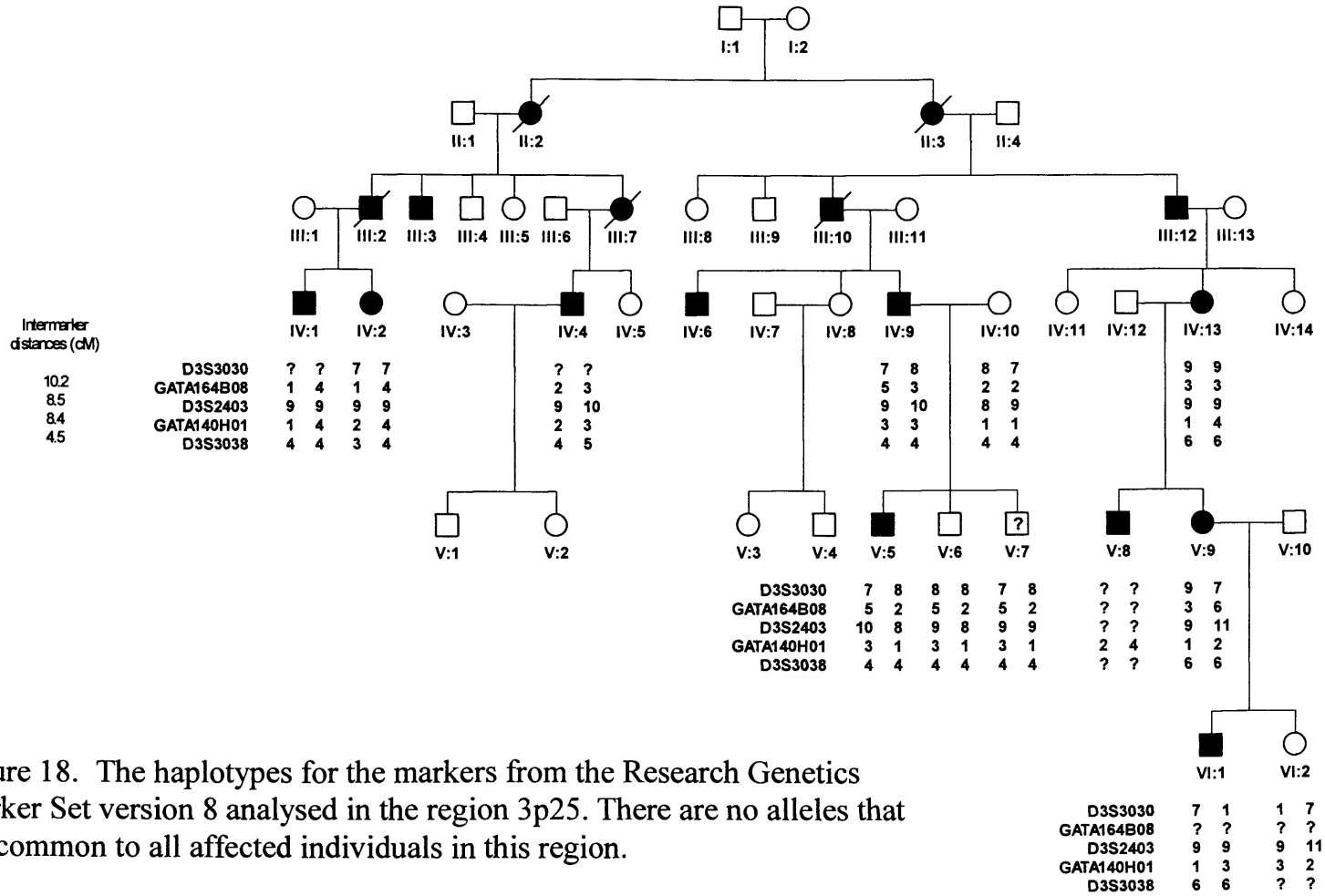


Figure 18. The haplotypes for the markers from the Research Genetics Marker Set version 8 analysed in the region 3p25. There are no alleles that are common to all affected individuals in this region.

Chromosome 8q22

Marker D8S1459 yielded positive lod scores with all three two point calculations. The MLINK analysis of affecteds and unaffecteds, with V:7 classed as affected, produced the highest two point lod score for this marker of 1.51 ($\theta = 0$). Linkage to the adjacent markers was excluded by the results of the two point lod score analyses. It can be seen from the haplotypes (figure 19) that all of the individuals who were genotyped, including four unaffected individuals, share allele 3 for D8S1459.

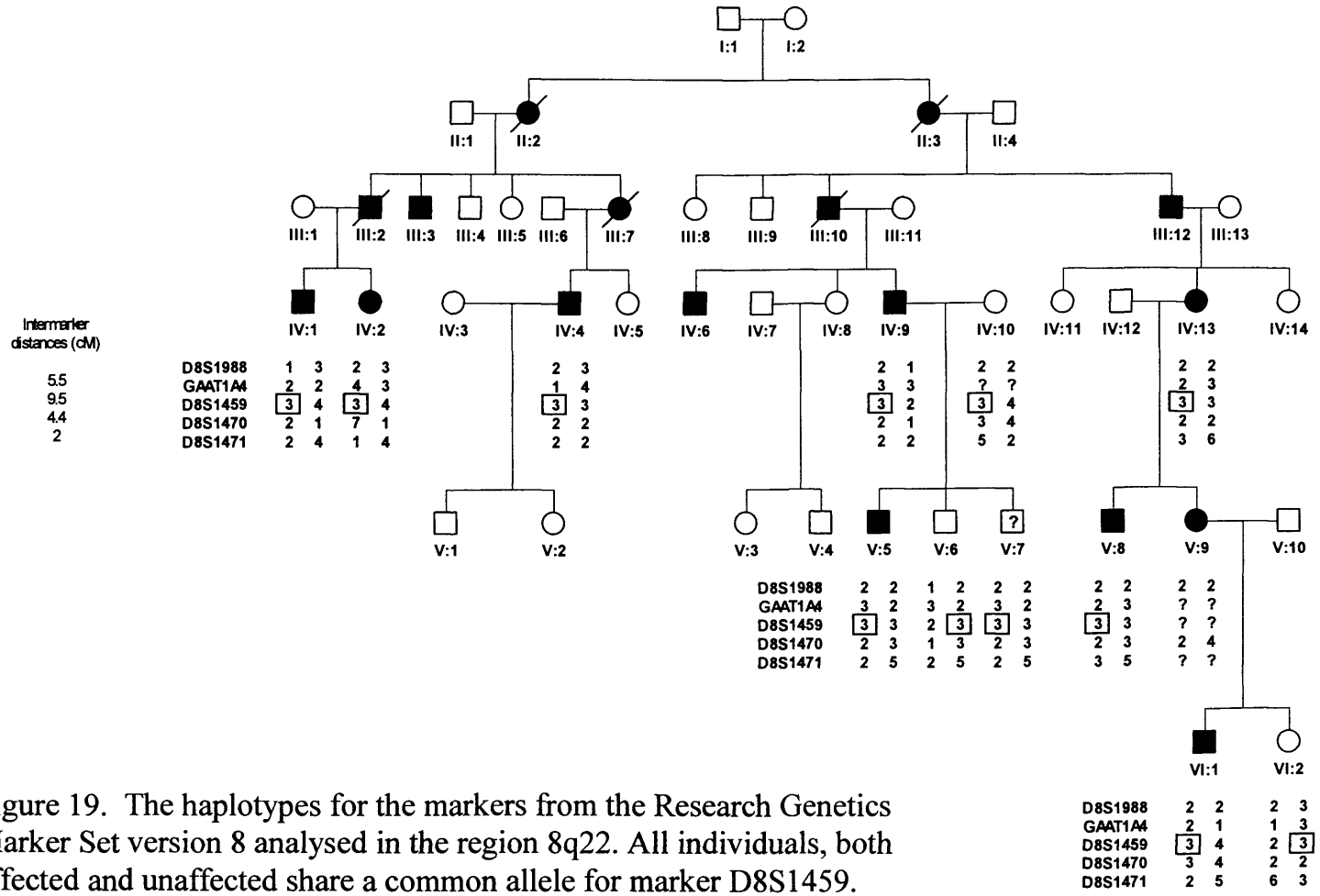


Figure 19. The haplotypes for the markers from the Research Genetics Marker Set version 8 analysed in the region 8q22. All individuals, both affected and unaffected share a common allele for marker D8S1459.

Chromosome 10q22

Using the LINKAGE software the maximum two point lod score for marker D10S1427 was 1.51 at a recombination fraction (θ) of 0.1. Unfortunately, attempted polymerase chain amplification using the markers adjacent to D10S1427 failed on repeated attempts. If candidate genes in other regions of interest are excluded by further investigation, it would be appropriate to analyse further microsatellite markers in this region.

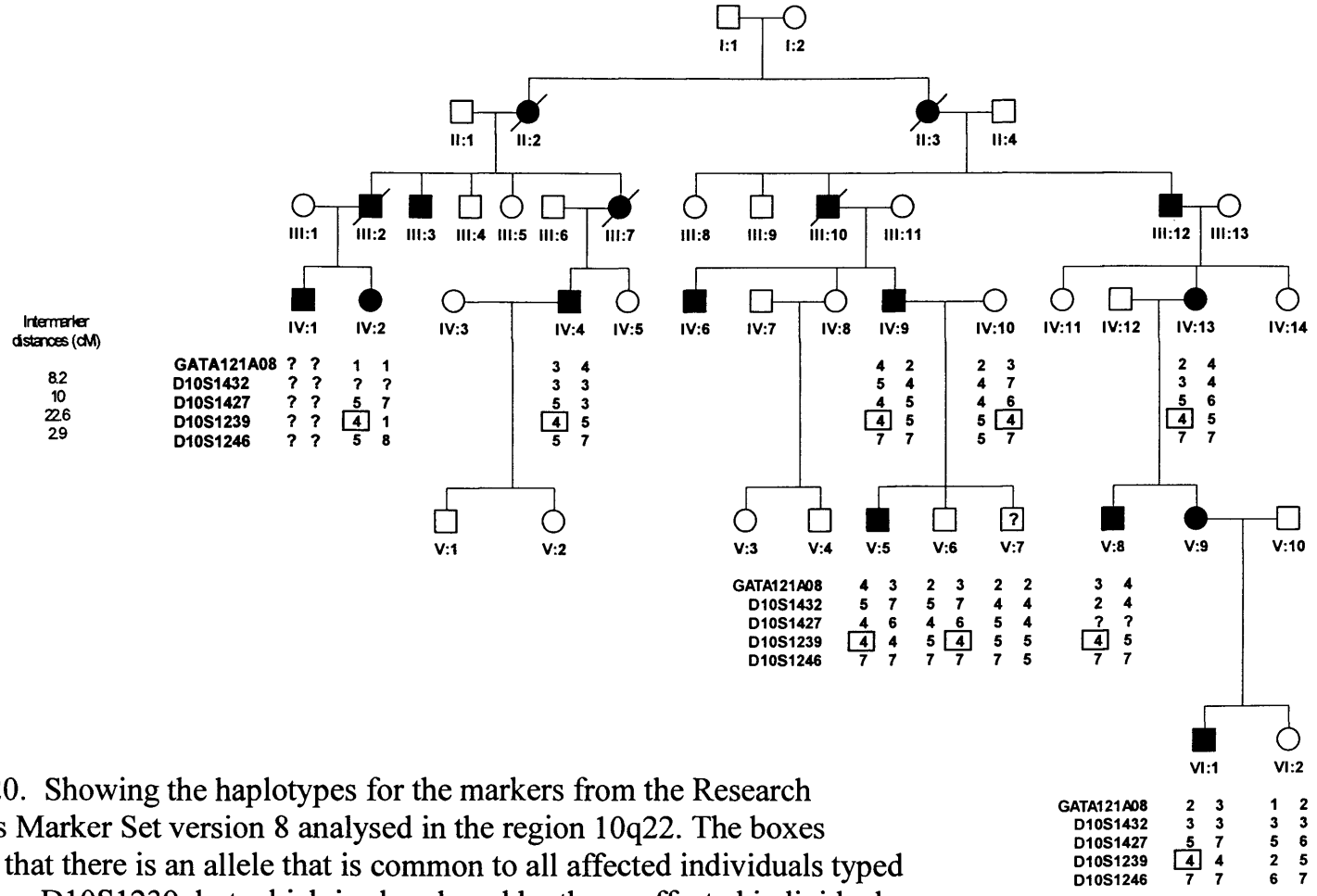


Figure 20. Showing the haplotypes for the markers from the Research Genetics Marker Set version 8 analysed in the region 10q22. The boxes indicate that there is an allele that is common to all affected individuals typed for marker D10S1239, but which is also shared by the unaffected individuals IV:10 and V:6.

5.2.3 Genome-wide multipoint linkage analysis

The data from genotyping family R with the Research Genetics screening set version 8.0 were used to calculate multipoint lod scores for each chromosome using the GENEHUNTER version 2 program (www.hgmp.mrc.ac.uk). The disease gene frequency was defined as 0.0002 with full penetrance.

Affected individuals only were included in the analysis, and since the affection status of individual V:7 had been called into question by this time and he was classed as unaffected in the multipoint analyses.

A summary of the most notable multipoint lod scores is shown in table 34, and a plot of the multipoint lod scores across each chromosome is shown in figures 21 to 43.

Table 34. Summary of the highest lod scores obtained by multipoint analysis

Chromosomal band location	Maximum Multipoint Lod Score using GENEHUNTER analysis (V:7 unknown)	Maximum Two Point Lod Score using GENEHUNTER analysis (V:7 unknown)	Closest Marker analysed to multipoint peak
2q32 – 2q37, 237cM	3.03	1.22	D1S1649
5q34-5q35, 201cM	2.86	3.18	D5S1713
3q25, 185cM	2.05	2.86	D3S4531
8p21, 21cM	2.52	0.68	D8S1145
16p12, 36cM	1.70	1.34	D16S749
13q34 , 101cM	1.60	1.83	D13S895
10q22, 94cM	1.58	1.51 ($\theta = 0.1$)	D10S1427
11q25, 140cM	1.57	1.87	D11S2359
15q11 - 15q12, 17cM	1.49	-infinity	D15S217
14q23, 62cM	1.45	0.72	D14S588

GENEHUNTER multipoint linkage analysis results graphs

Figure 21. Chromosome 1 multipoint lod score results

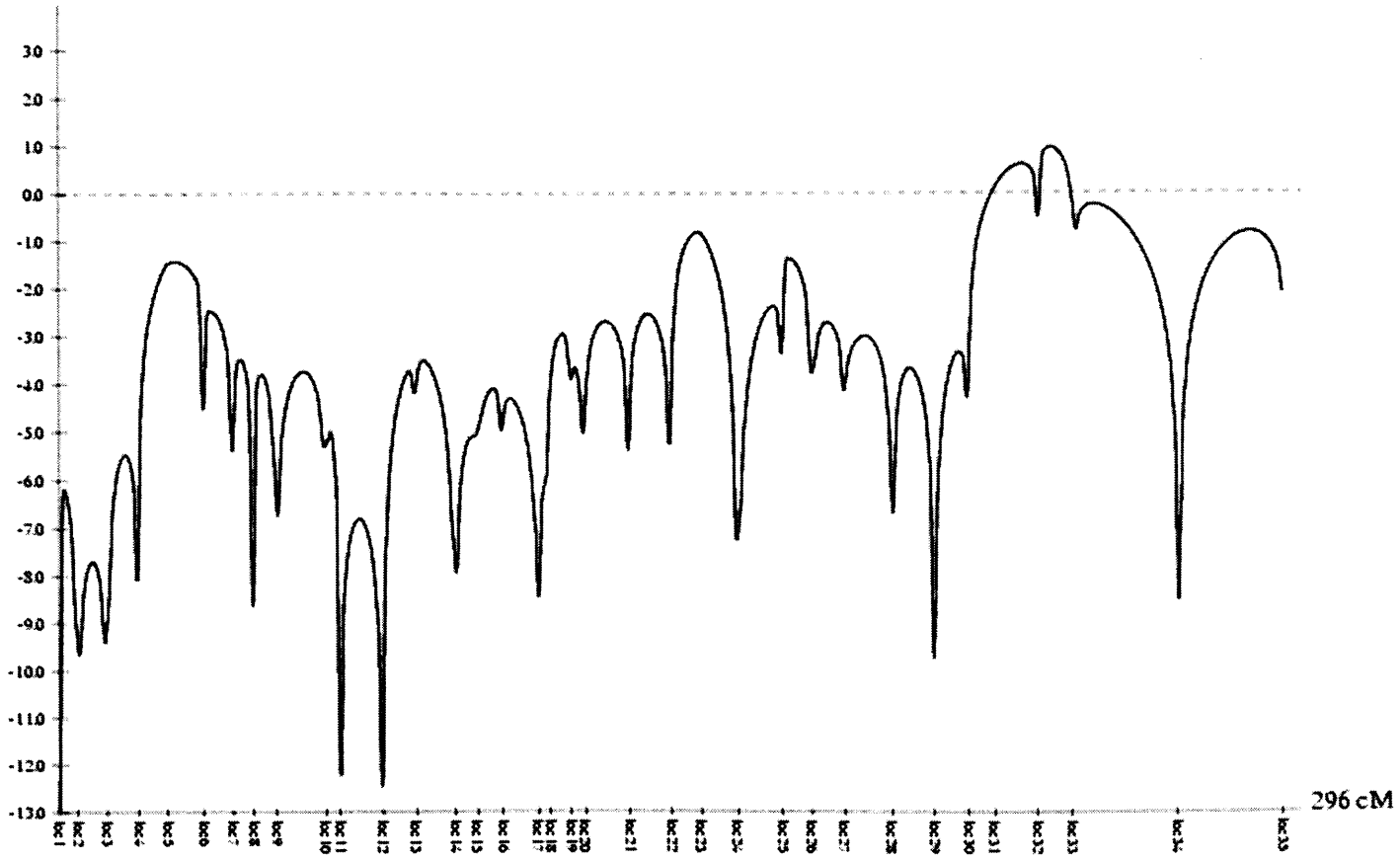


Figure22. Chromosome 2 multipoint lod score results

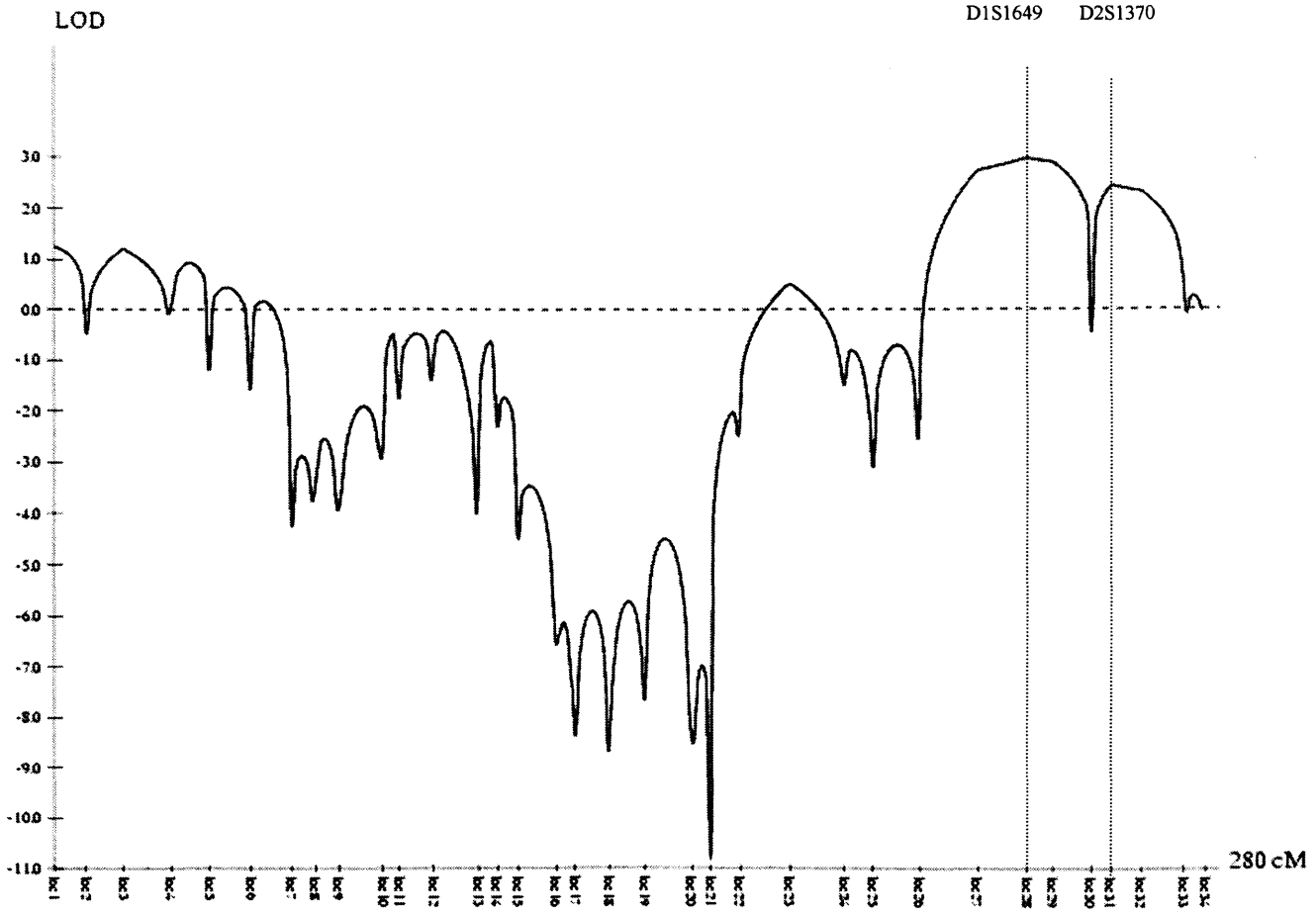


Figure 23. Multipoint lod scores for the Research Genetics markers used in the telomeric region of chromosome 2q

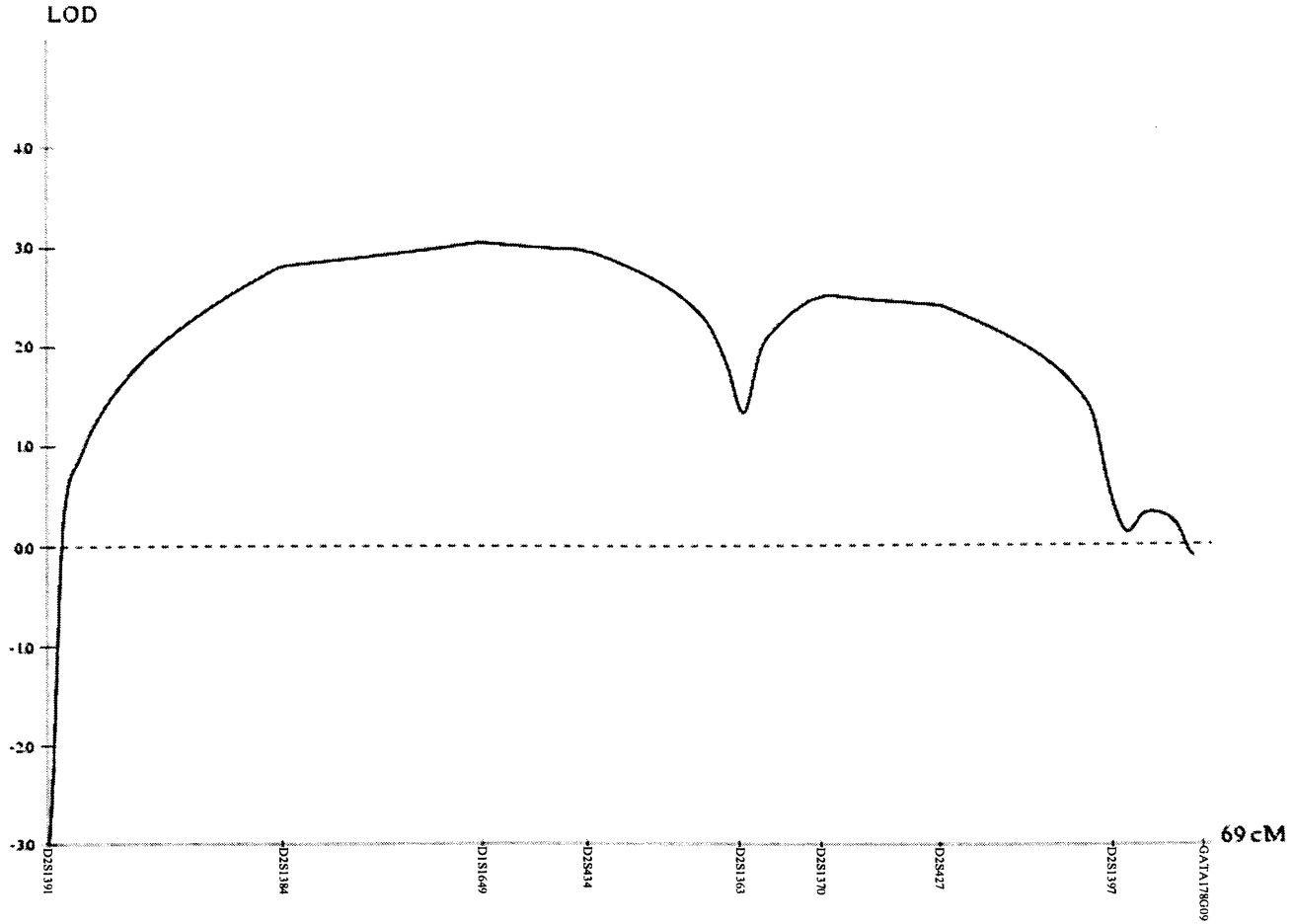


Figure 24. Chromosome 3 multipoint lod score results

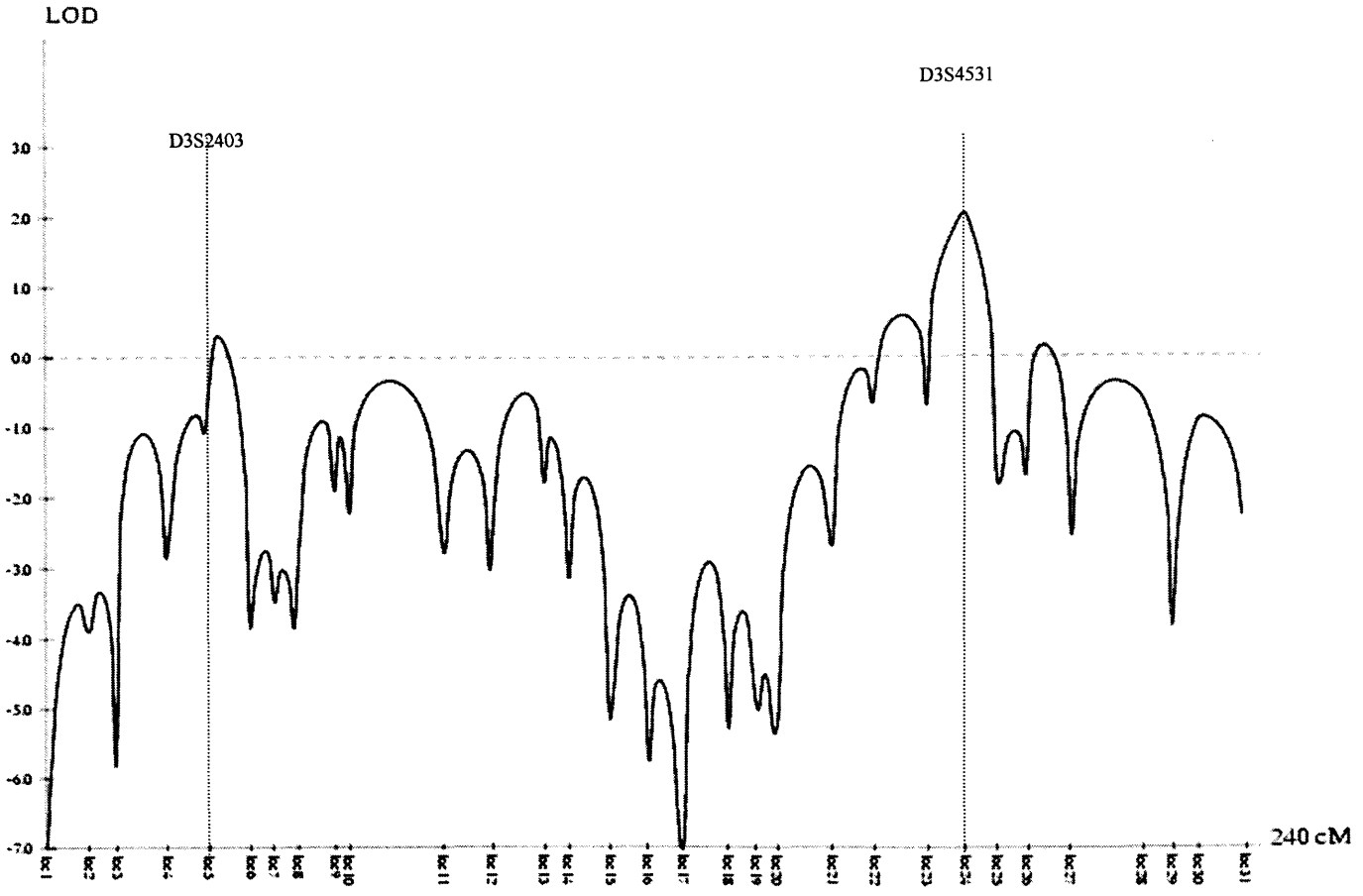


Figure 25. Chromosome 4 multipoint lod score results

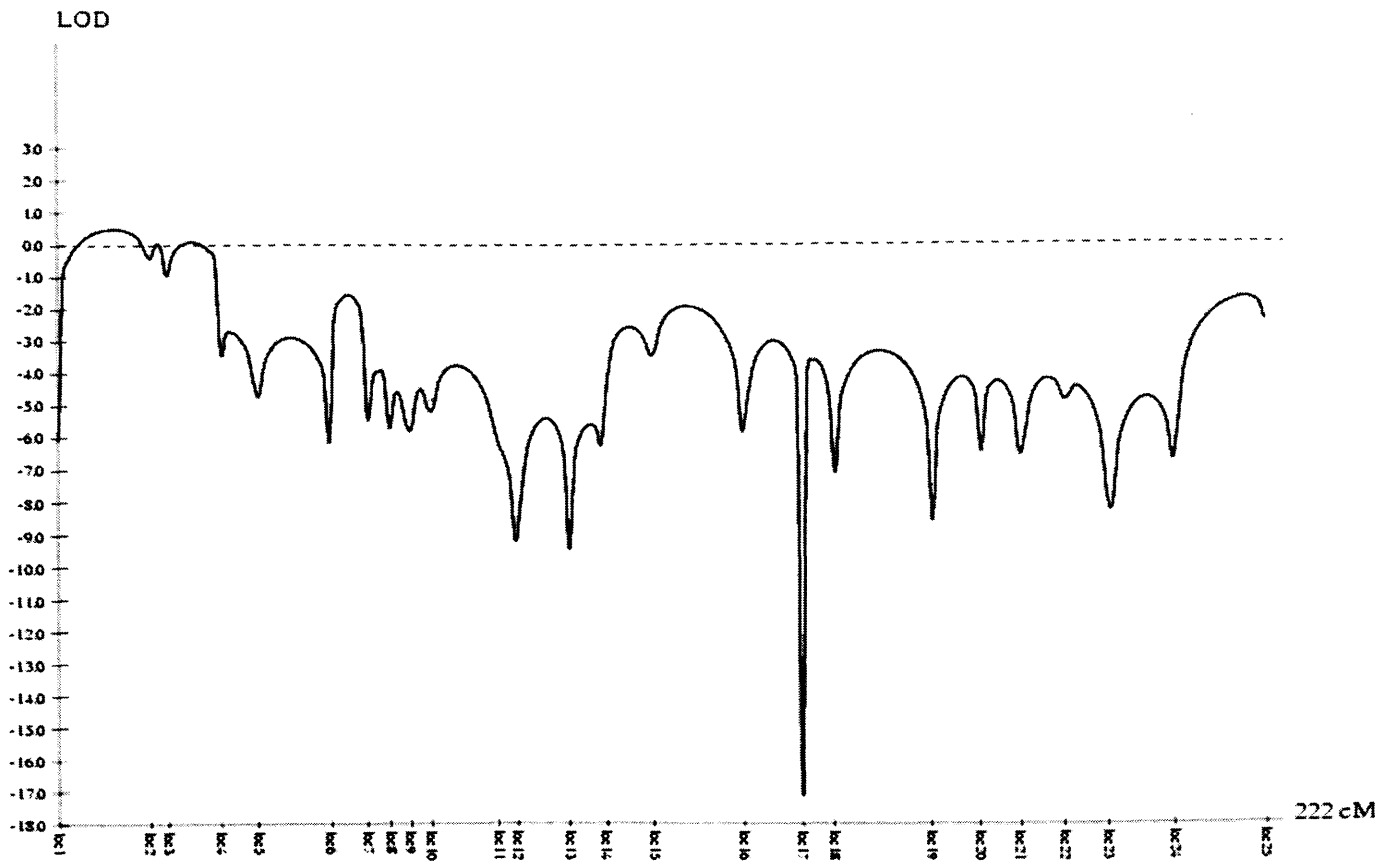


Figure 26. Chromosome 5 multipoint lod score results

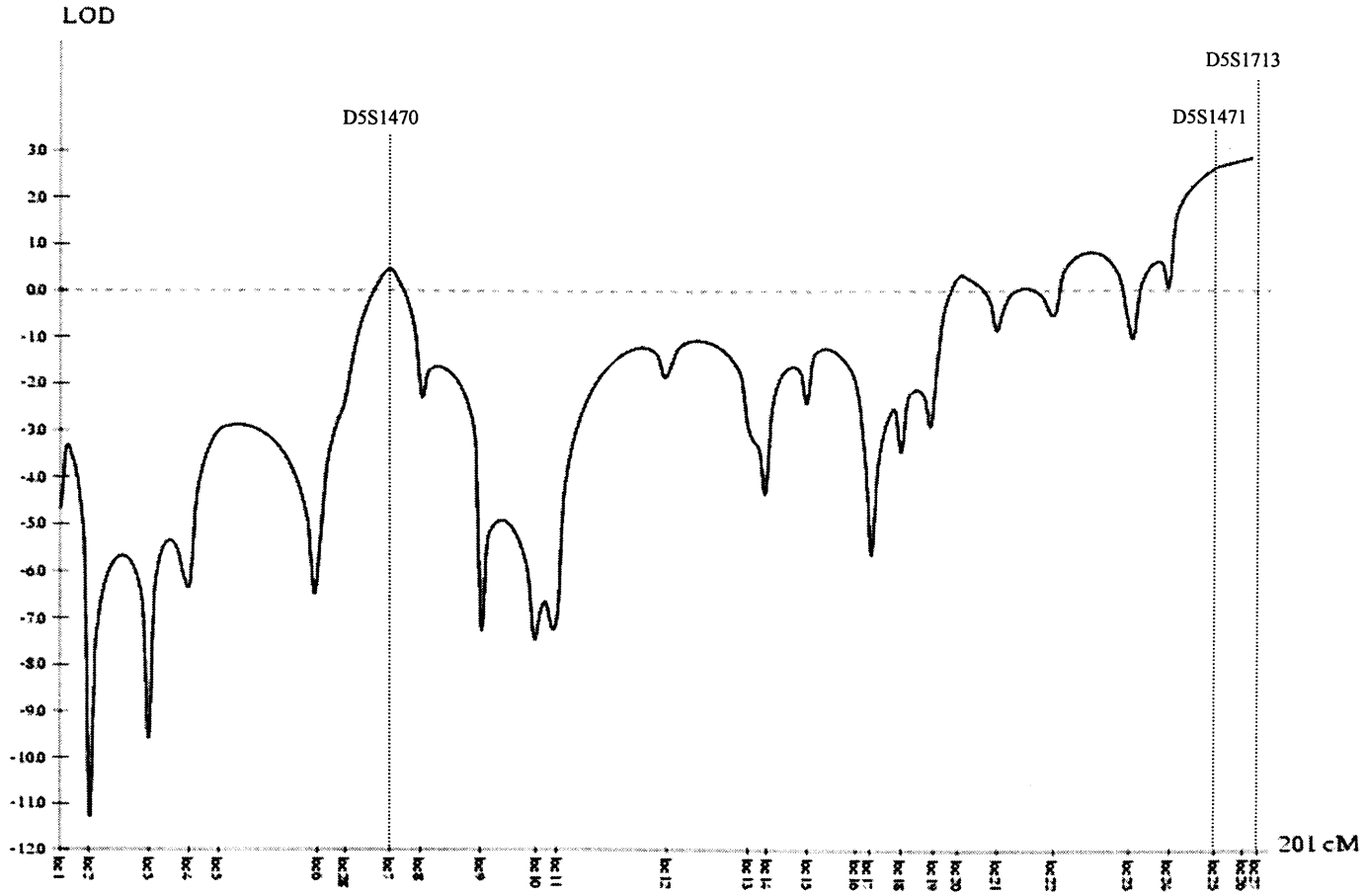


Figure 27. Chromosome 6 multipoint lod score results

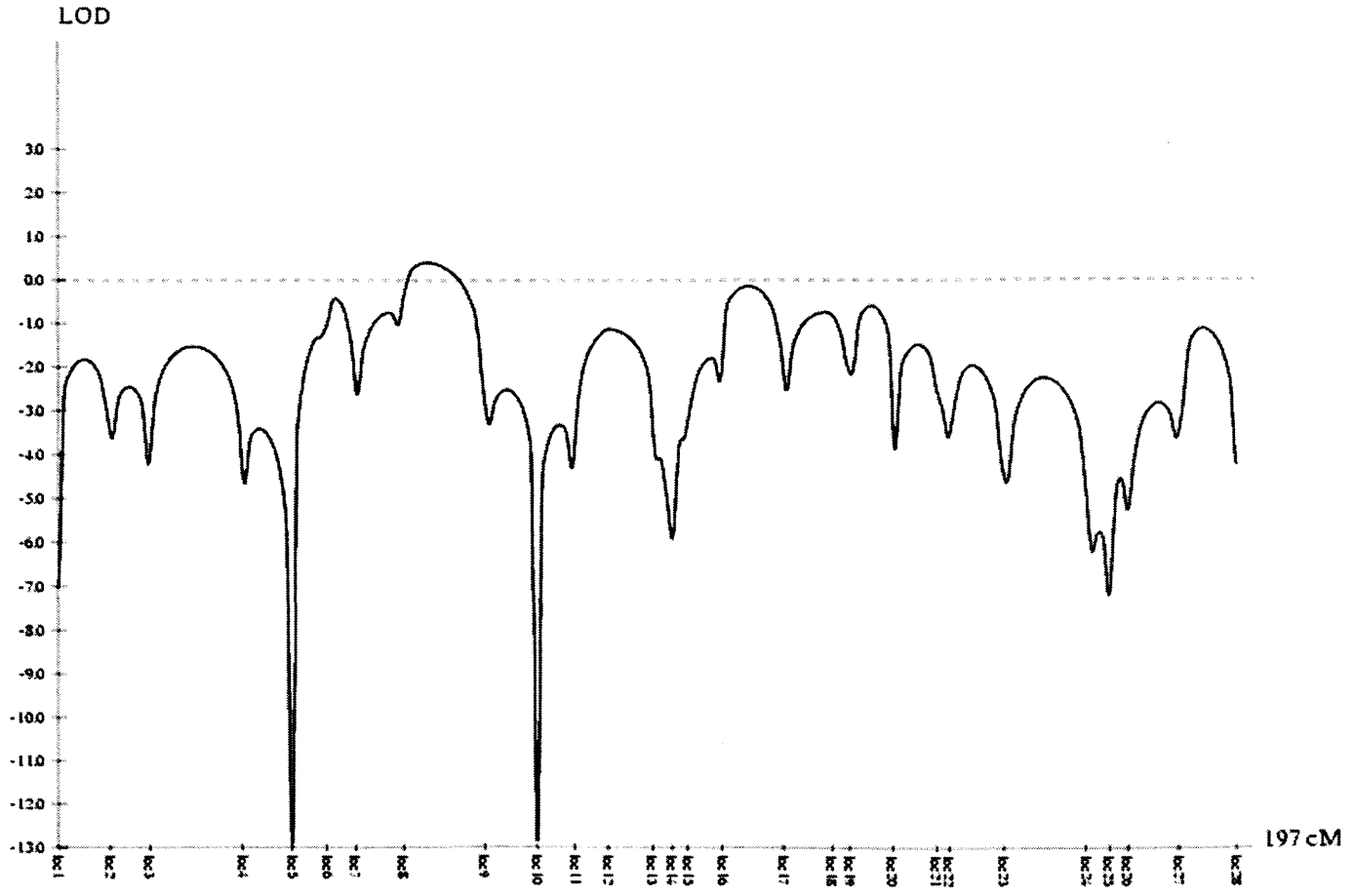


Figure 28. Chromosome 7 multipoint lod score results

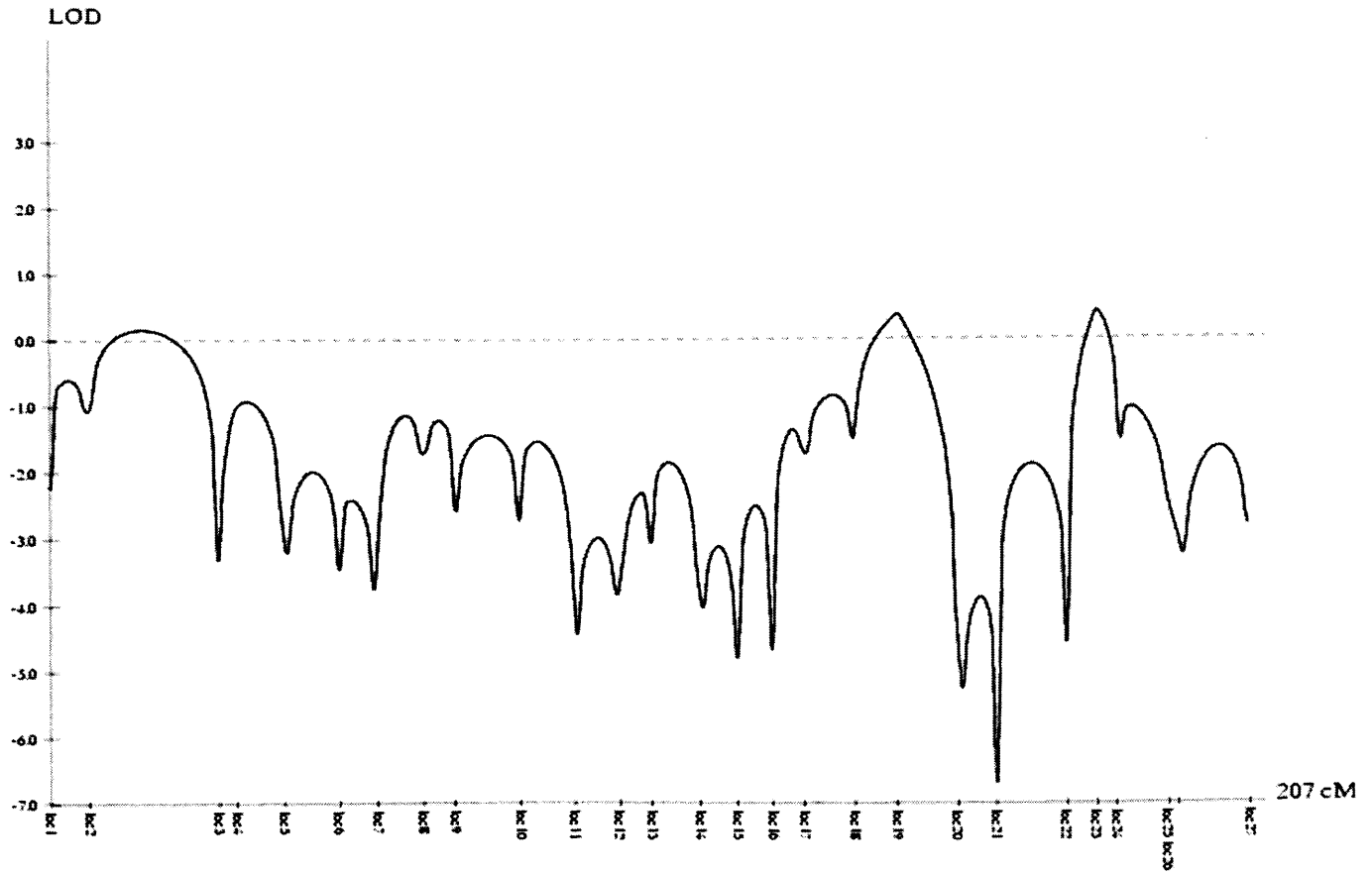


Figure 29. Chromosome 8 multipoint lod score results

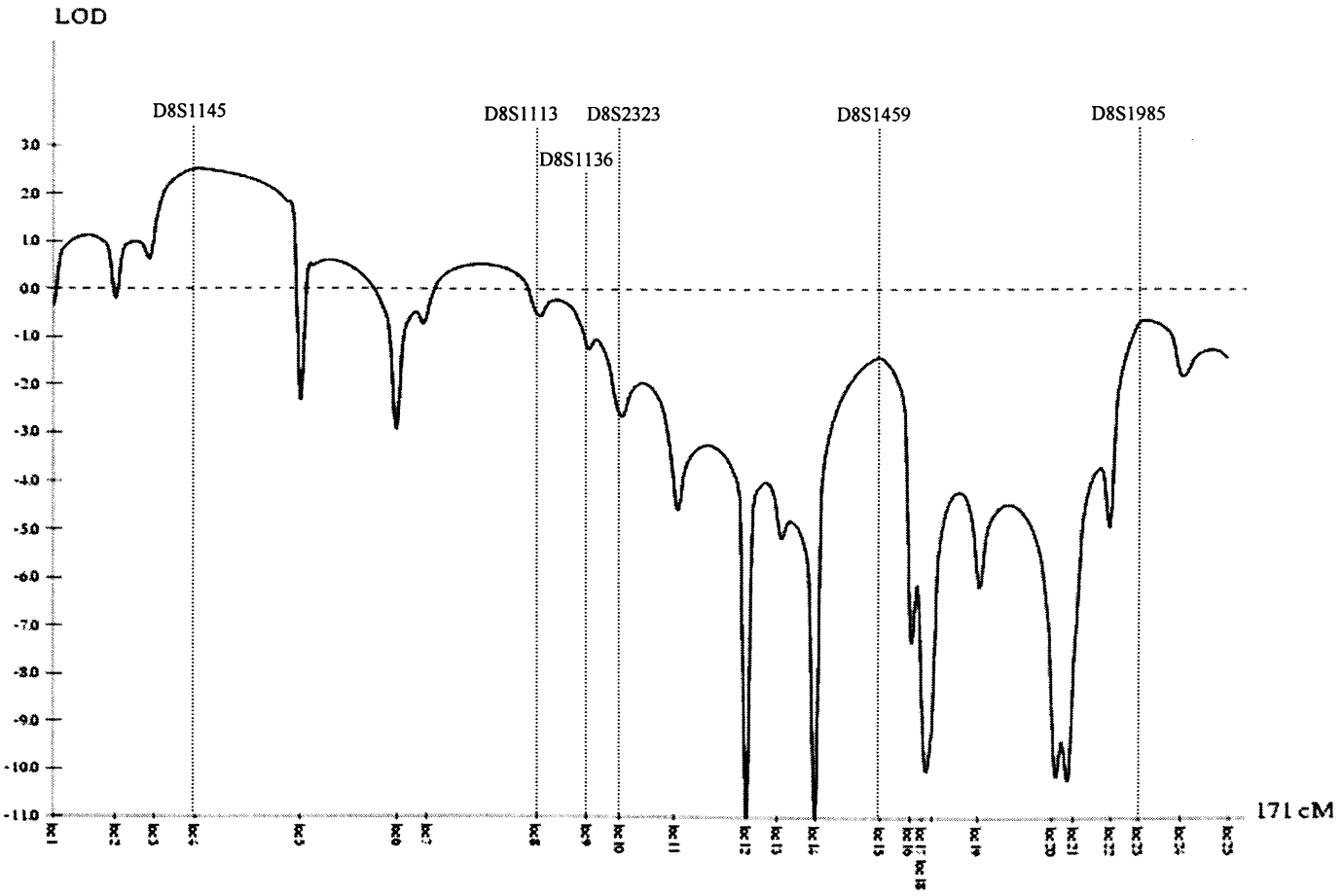


Figure 30. Chromosome 9 multipoint lod score results

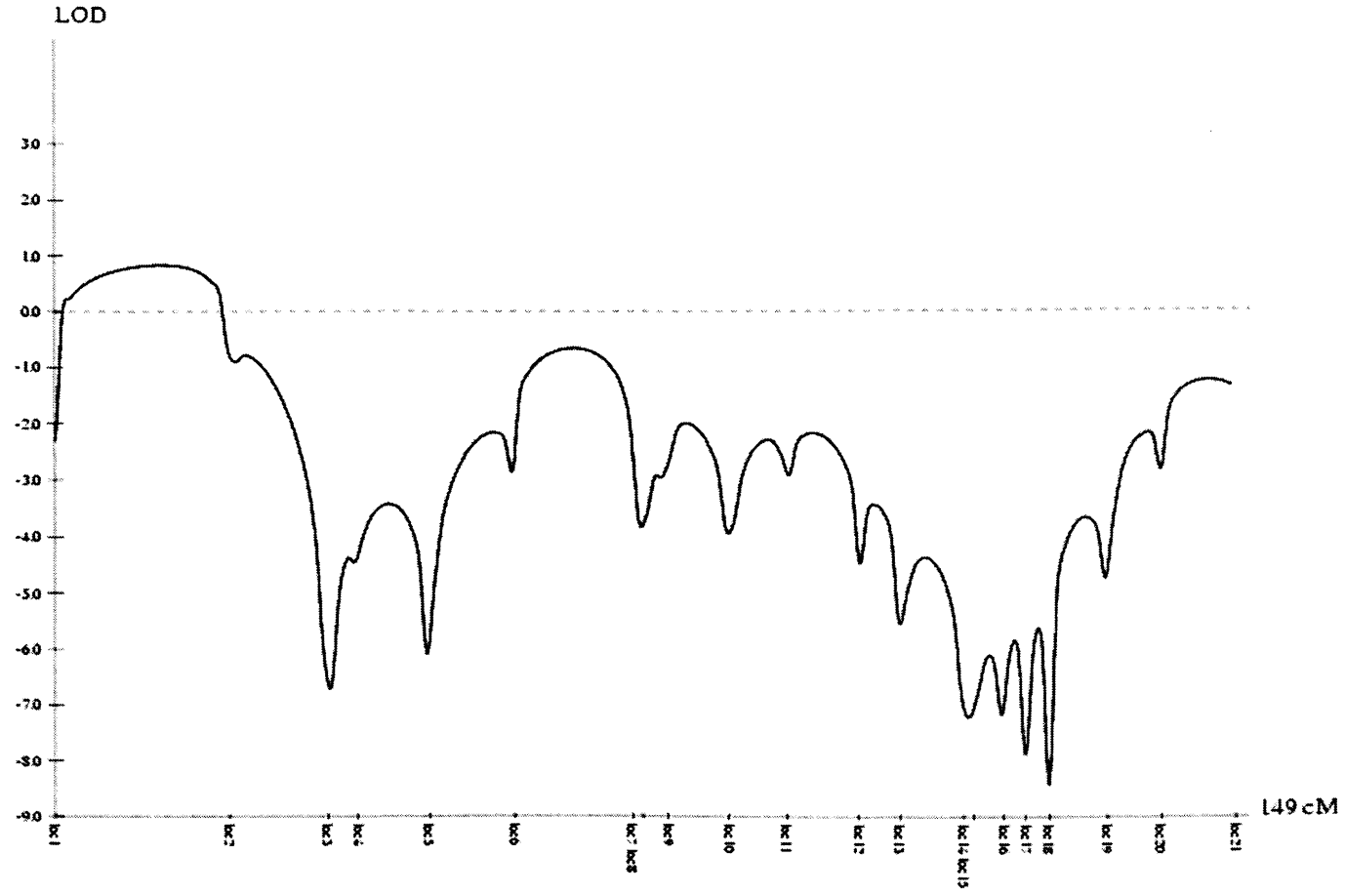


Figure 31. Chromosome 10 multipoint lod score results

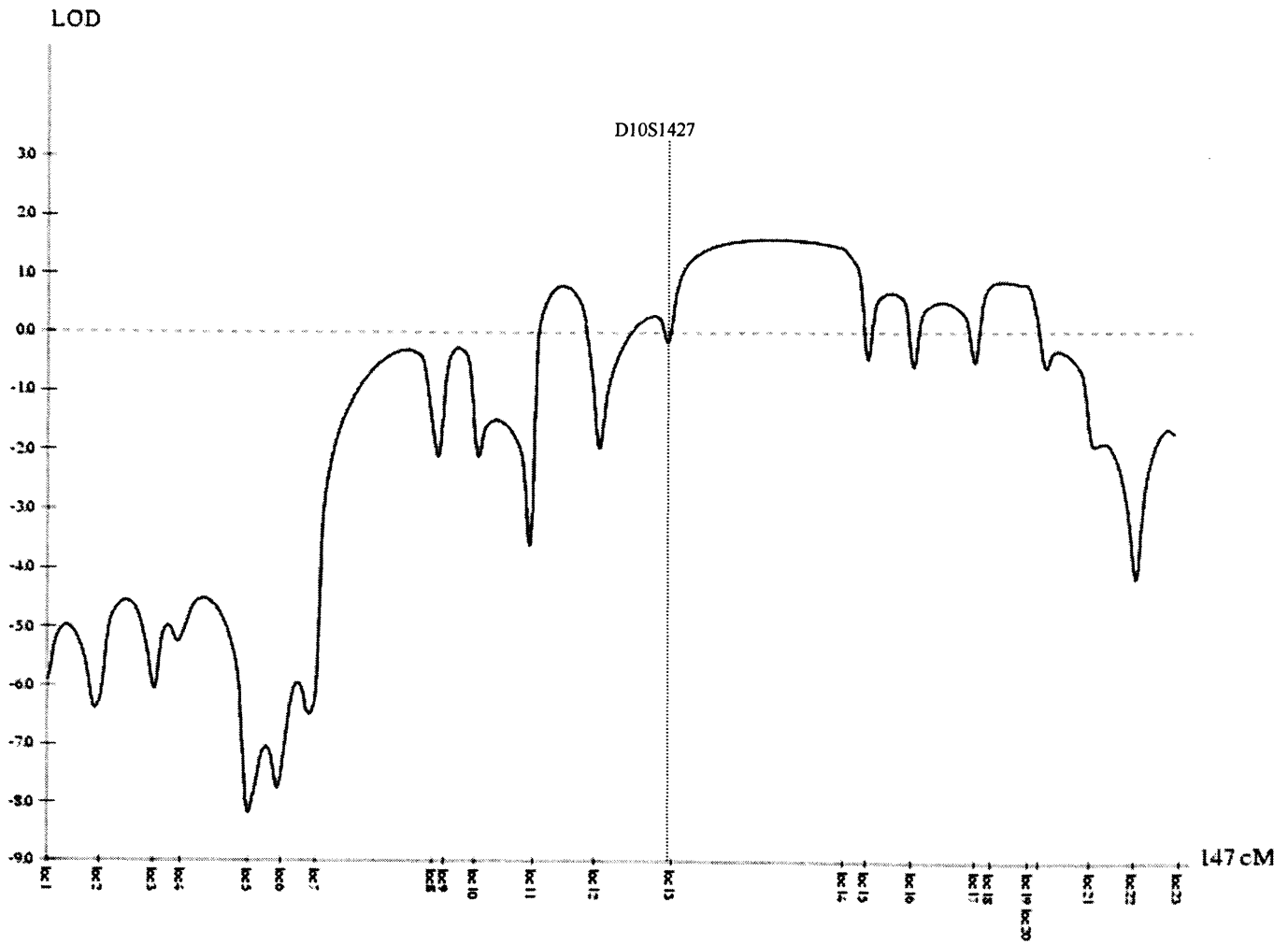


Figure 32. Chromosome 11 multipoint lod score results

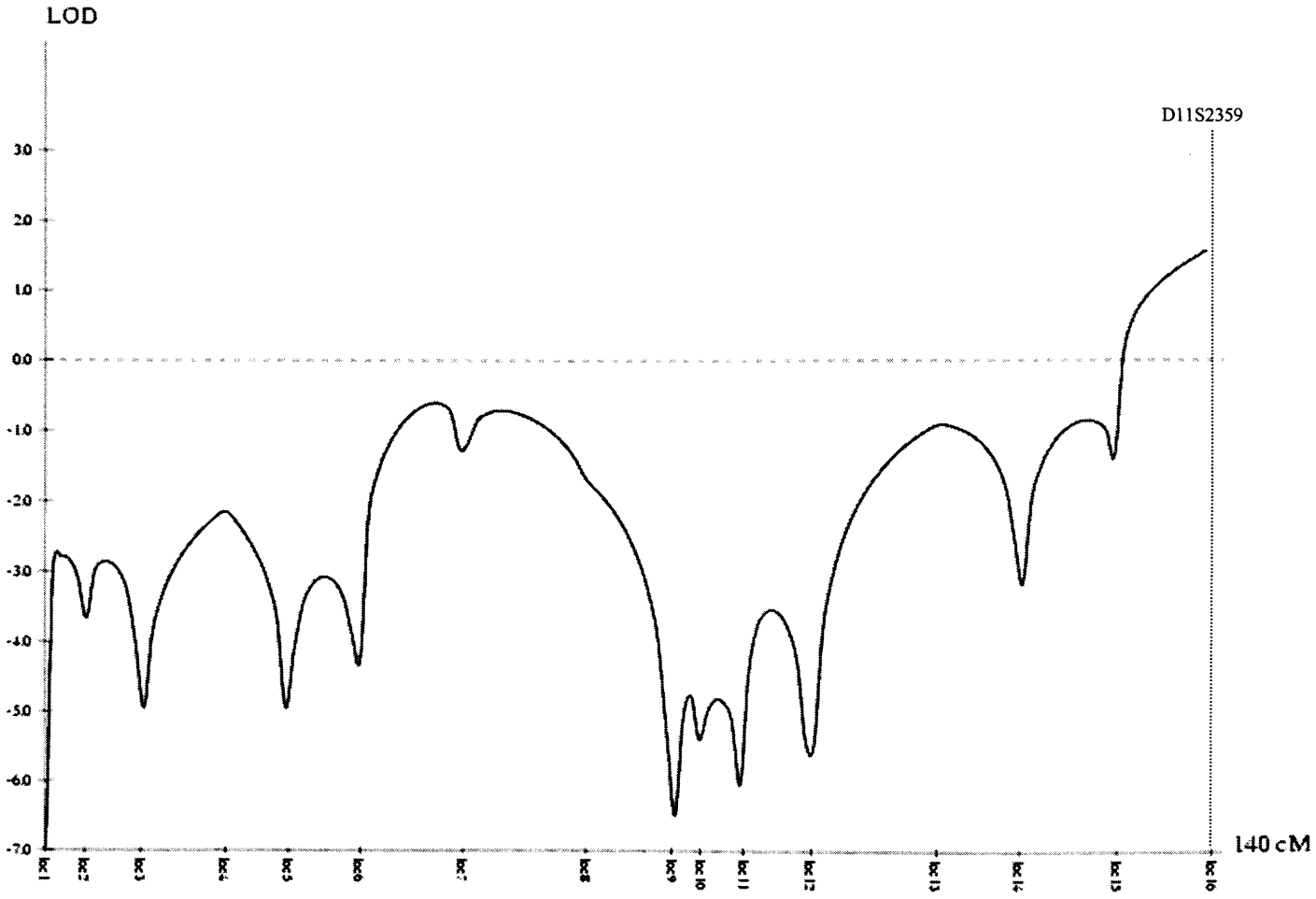


Figure 33. Chromosome 12 multipoint lod score results

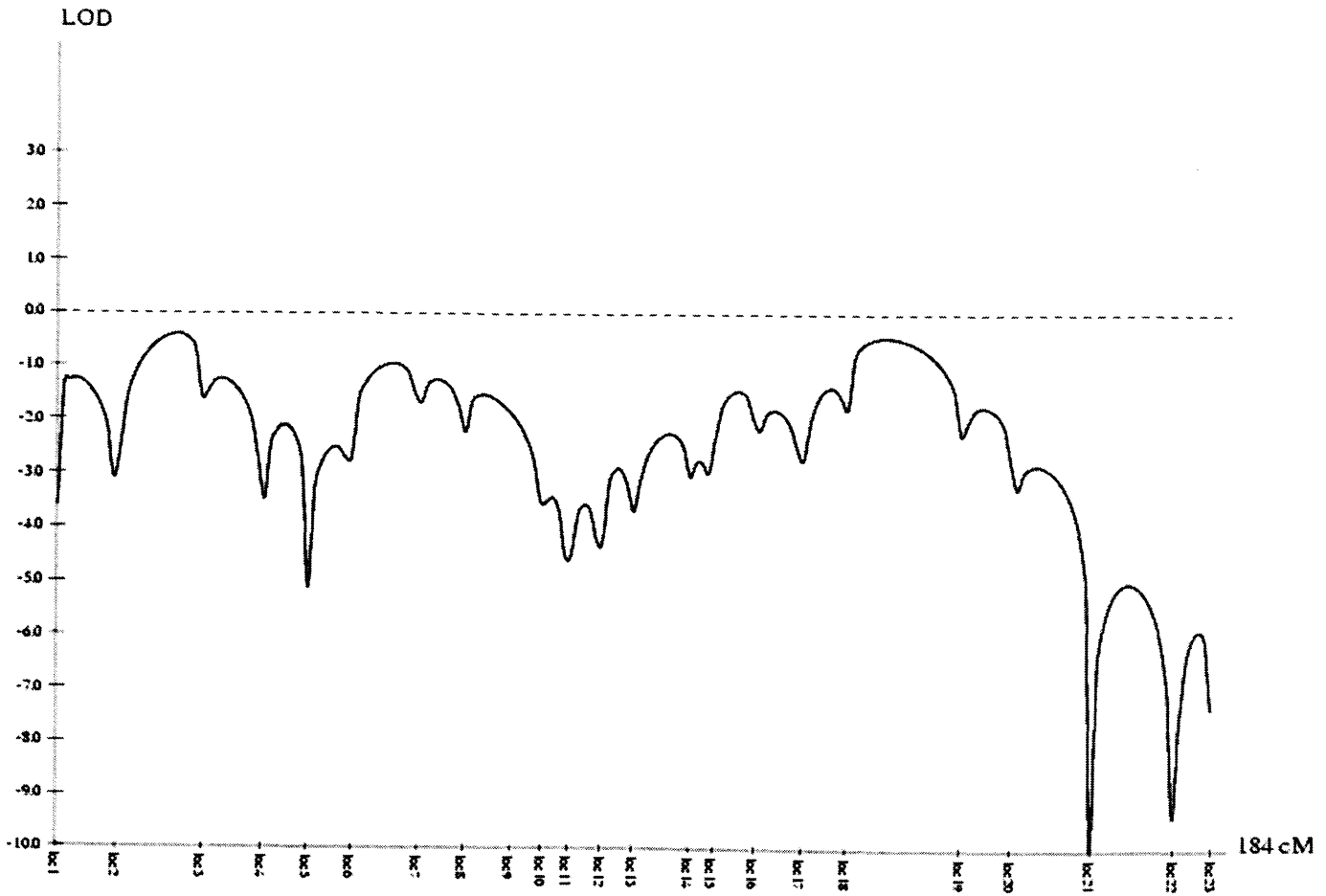


Figure 34. Chromosome 13 multipoint lod score results

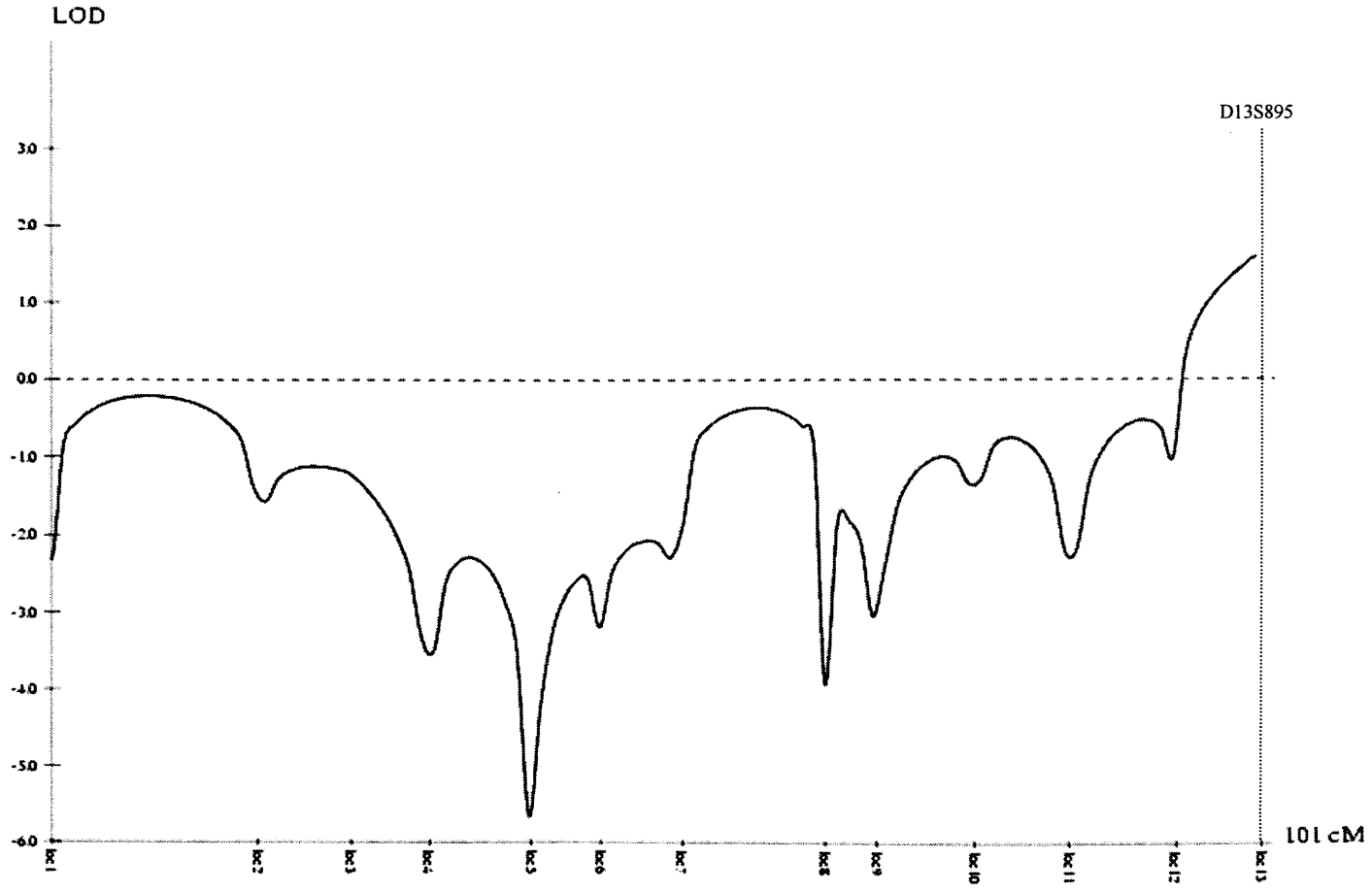


Figure 35. Chromosome 14 multipoint lod score results

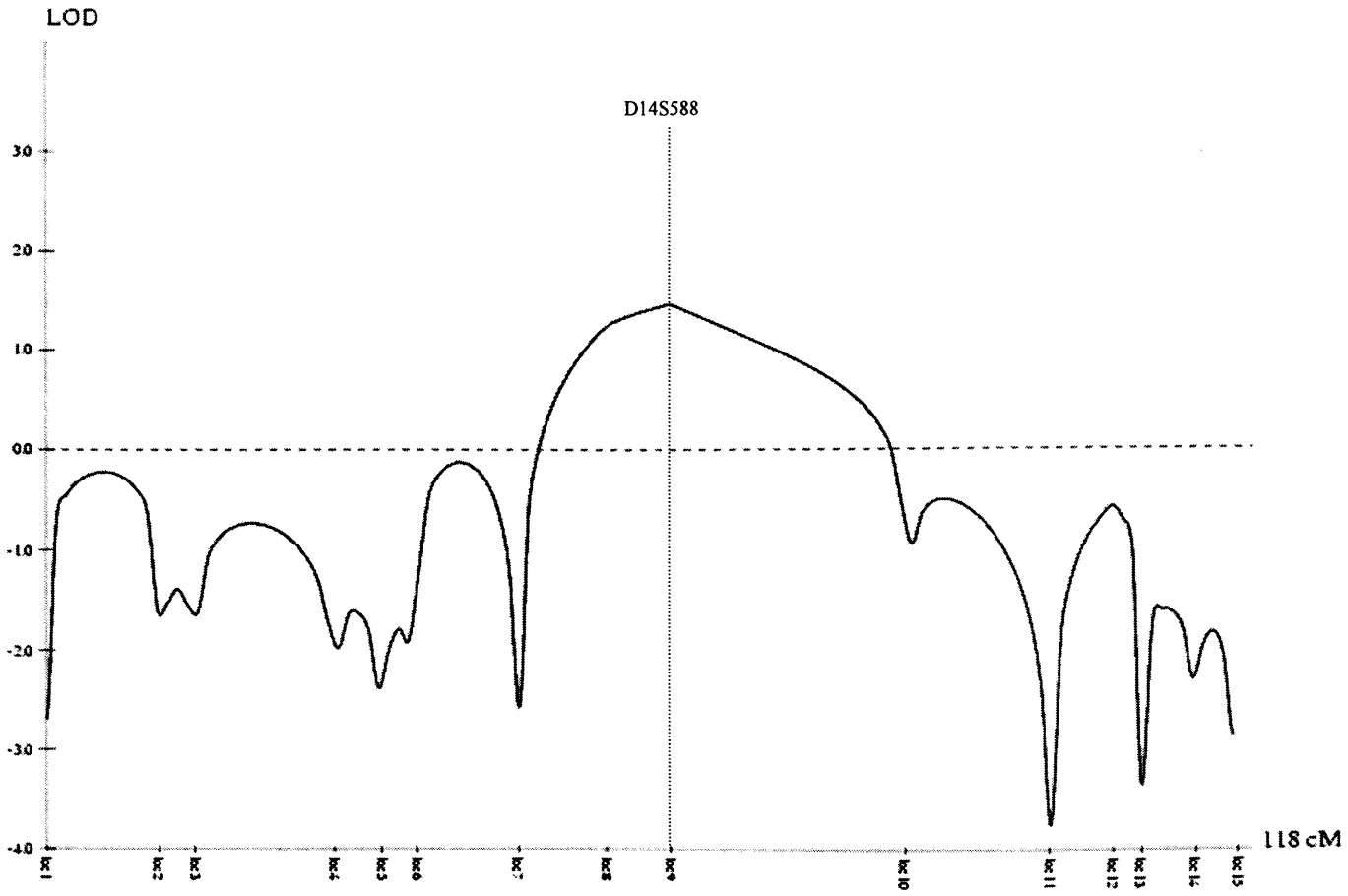


Figure 36. Chromosome 15 multipoint lod score results

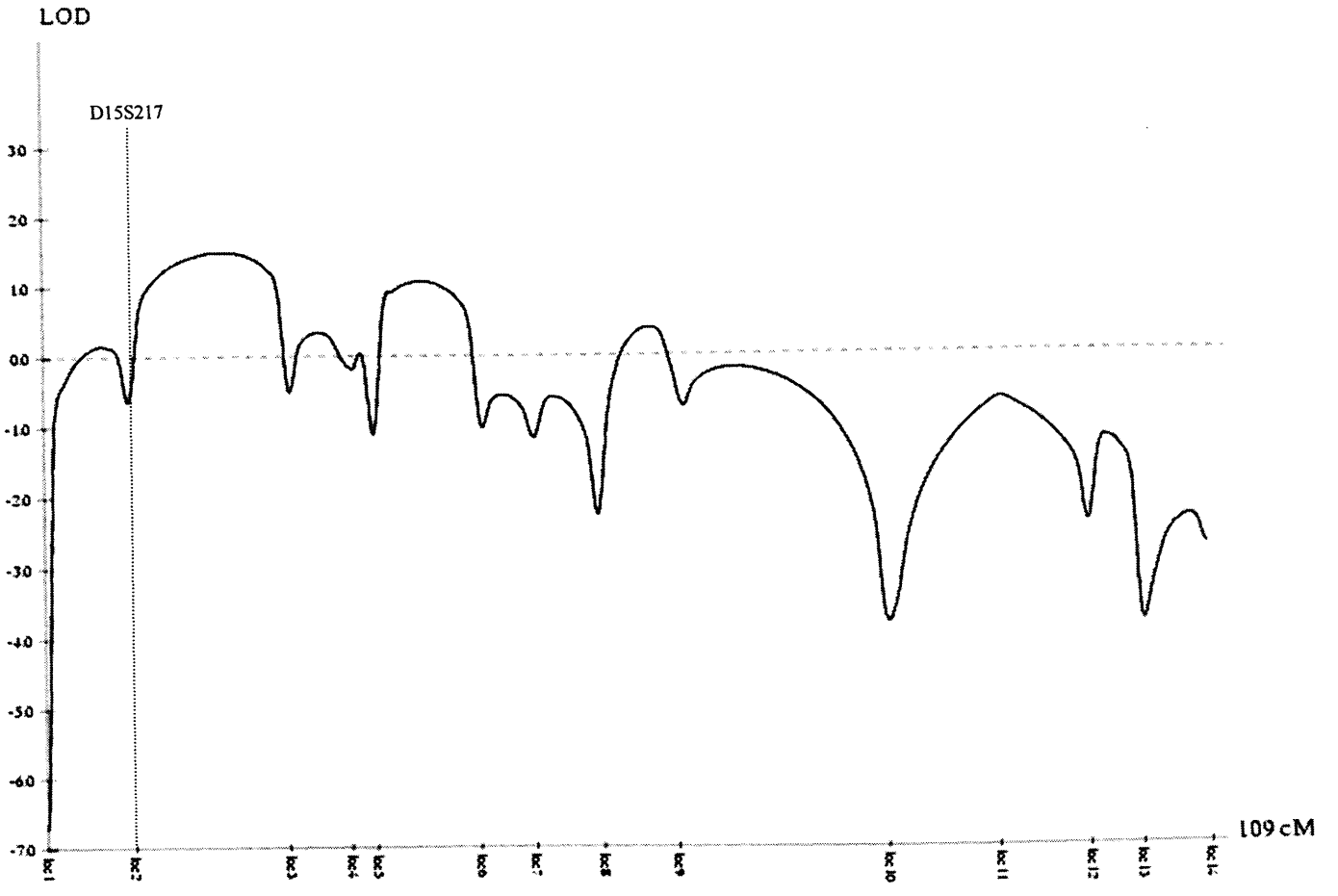


Figure 37. Chromosome 16 multipoint lod score results

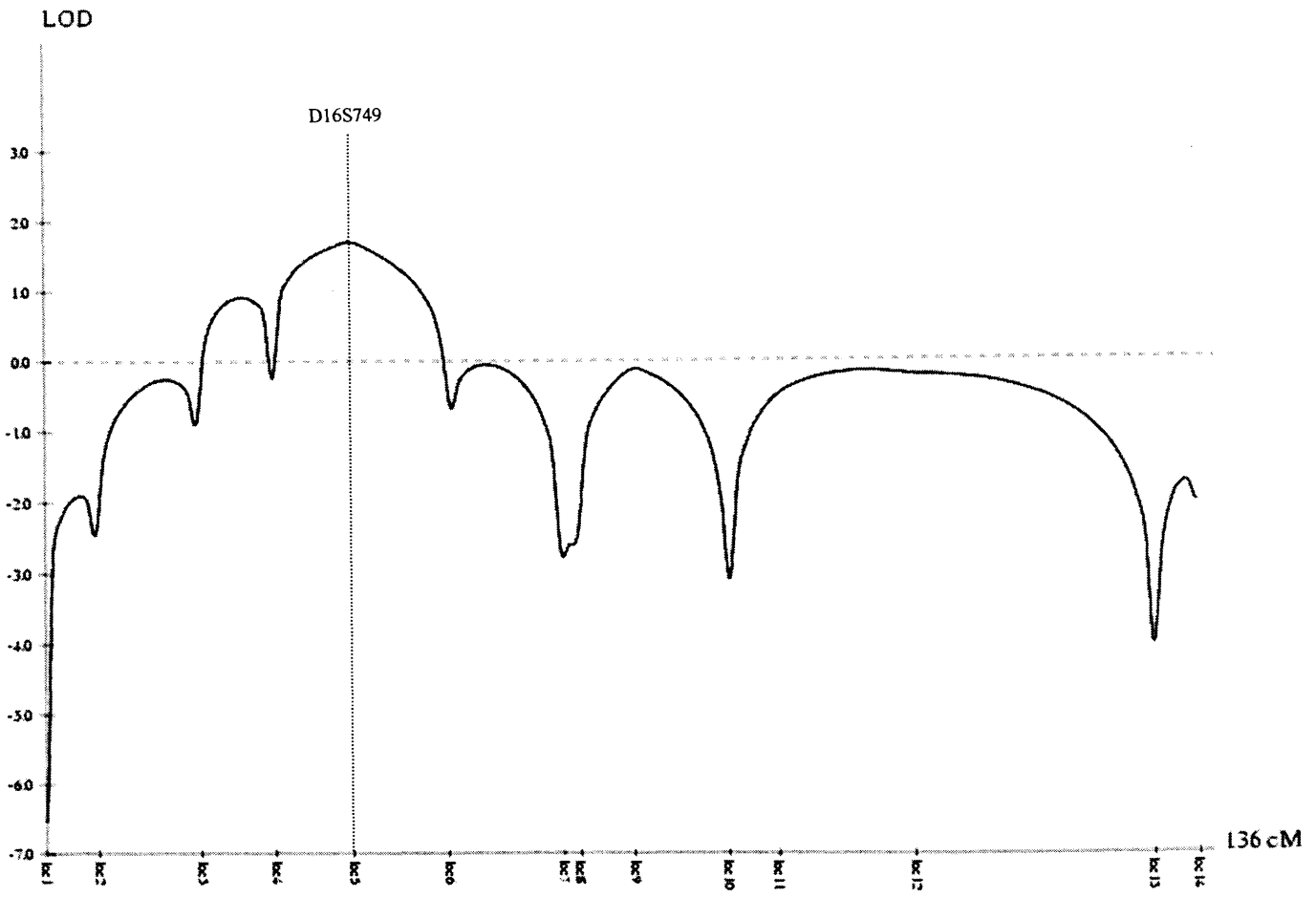


Figure 38. Chromosome 17 multipoint lod score results

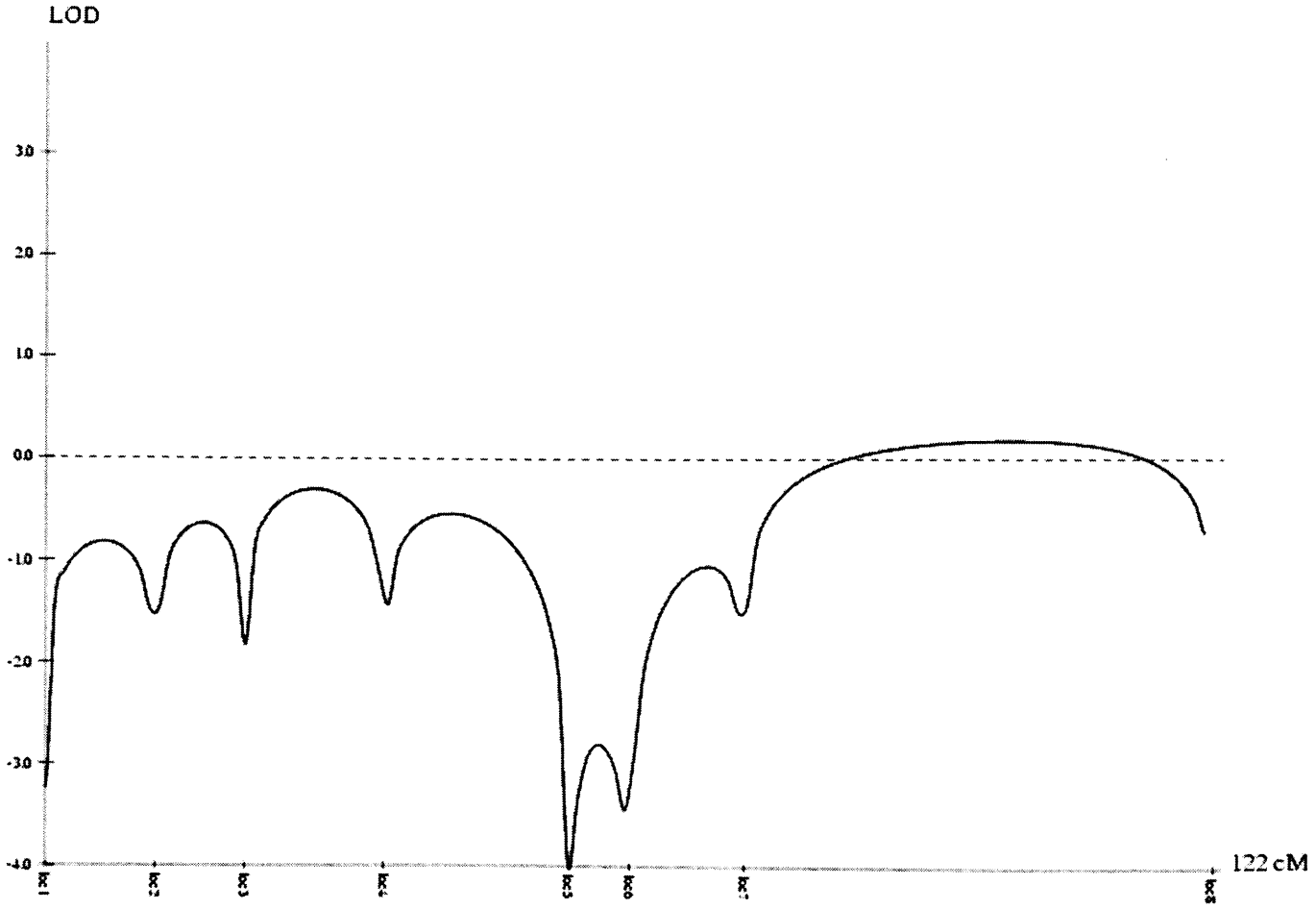


Figure 39. Chromosome 18 multipoint lod score results

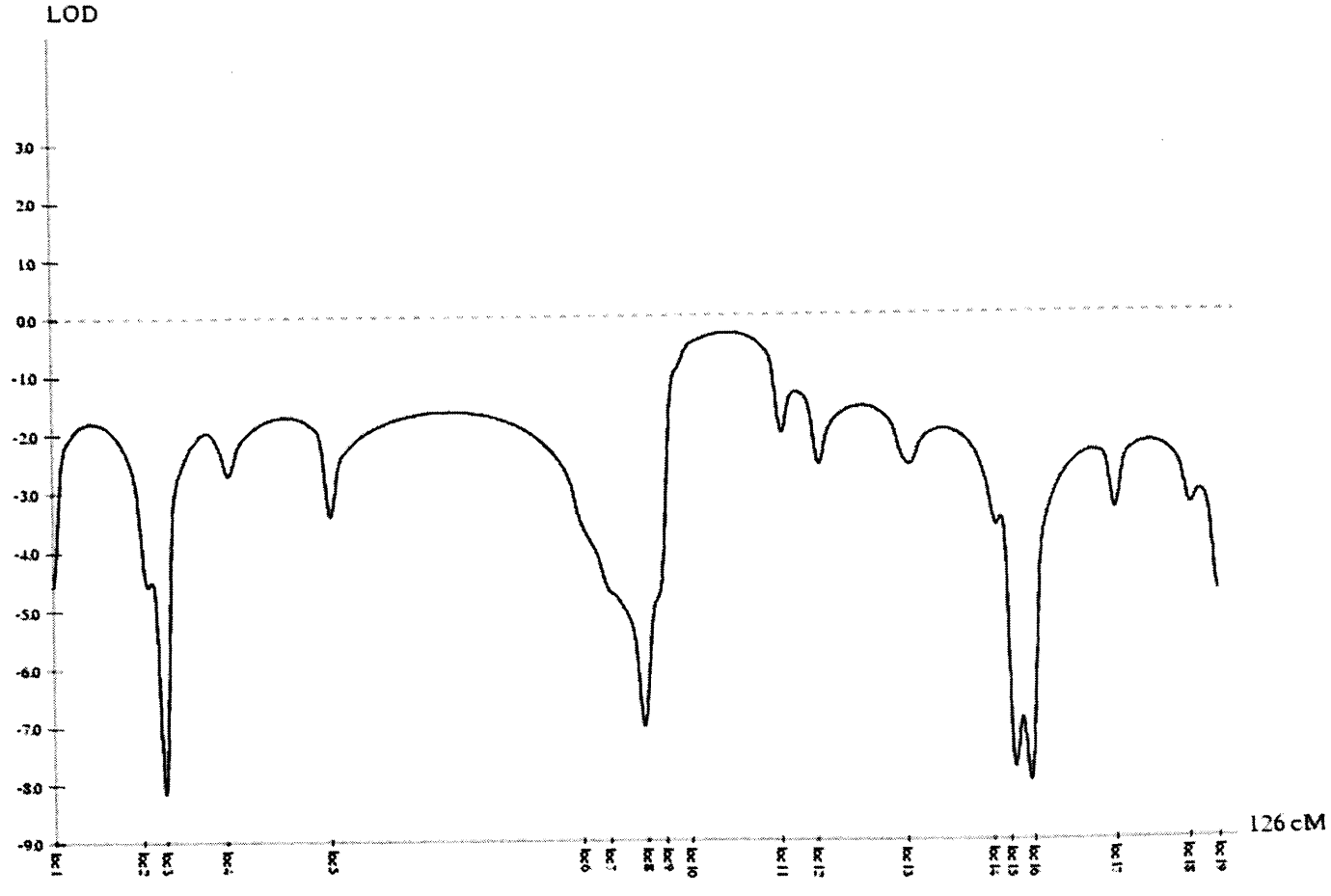


Figure 40. Chromosome 19 multipoint lod score results

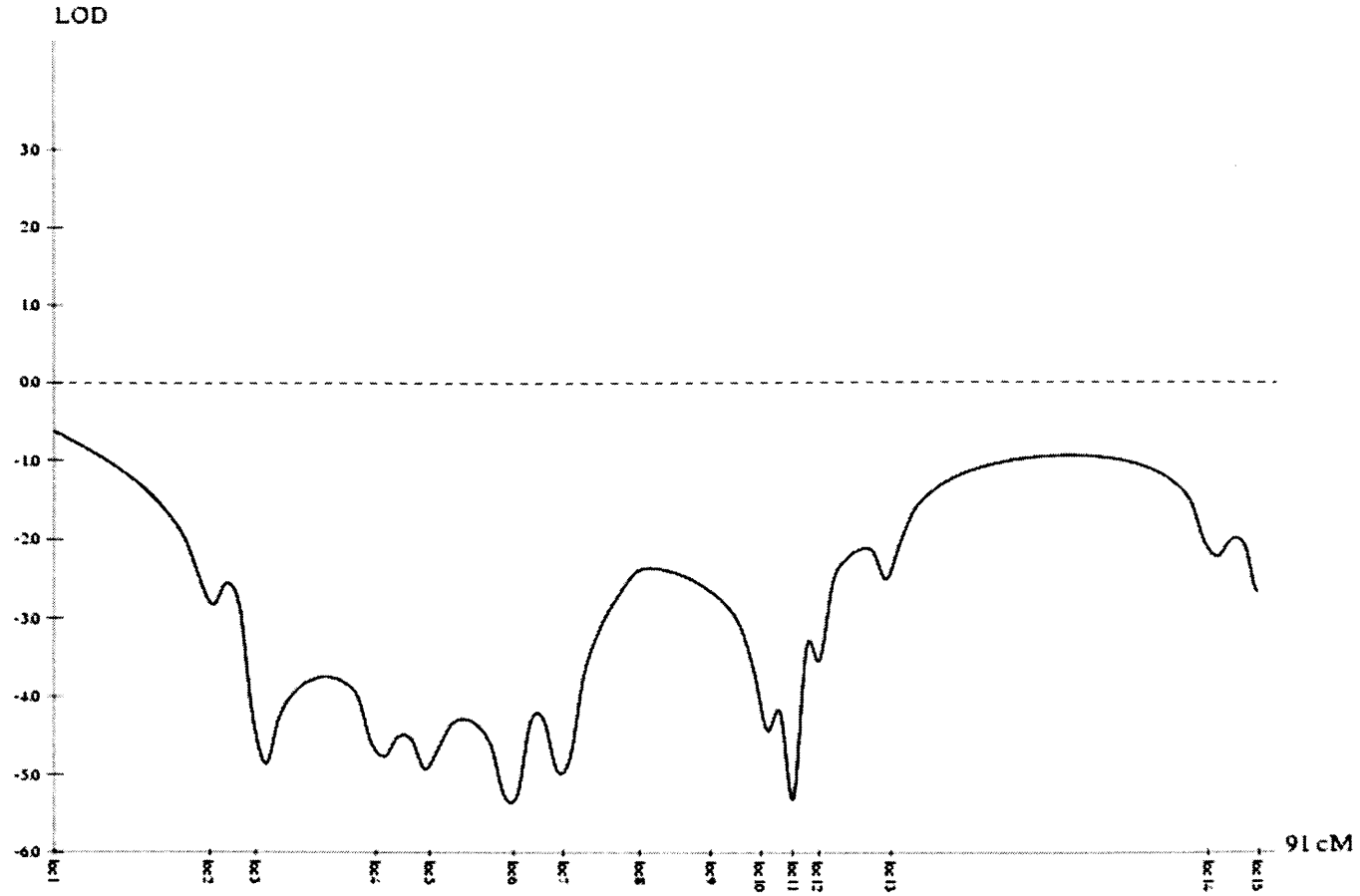


Figure 41. Chromosome 20 multipoint lod score results

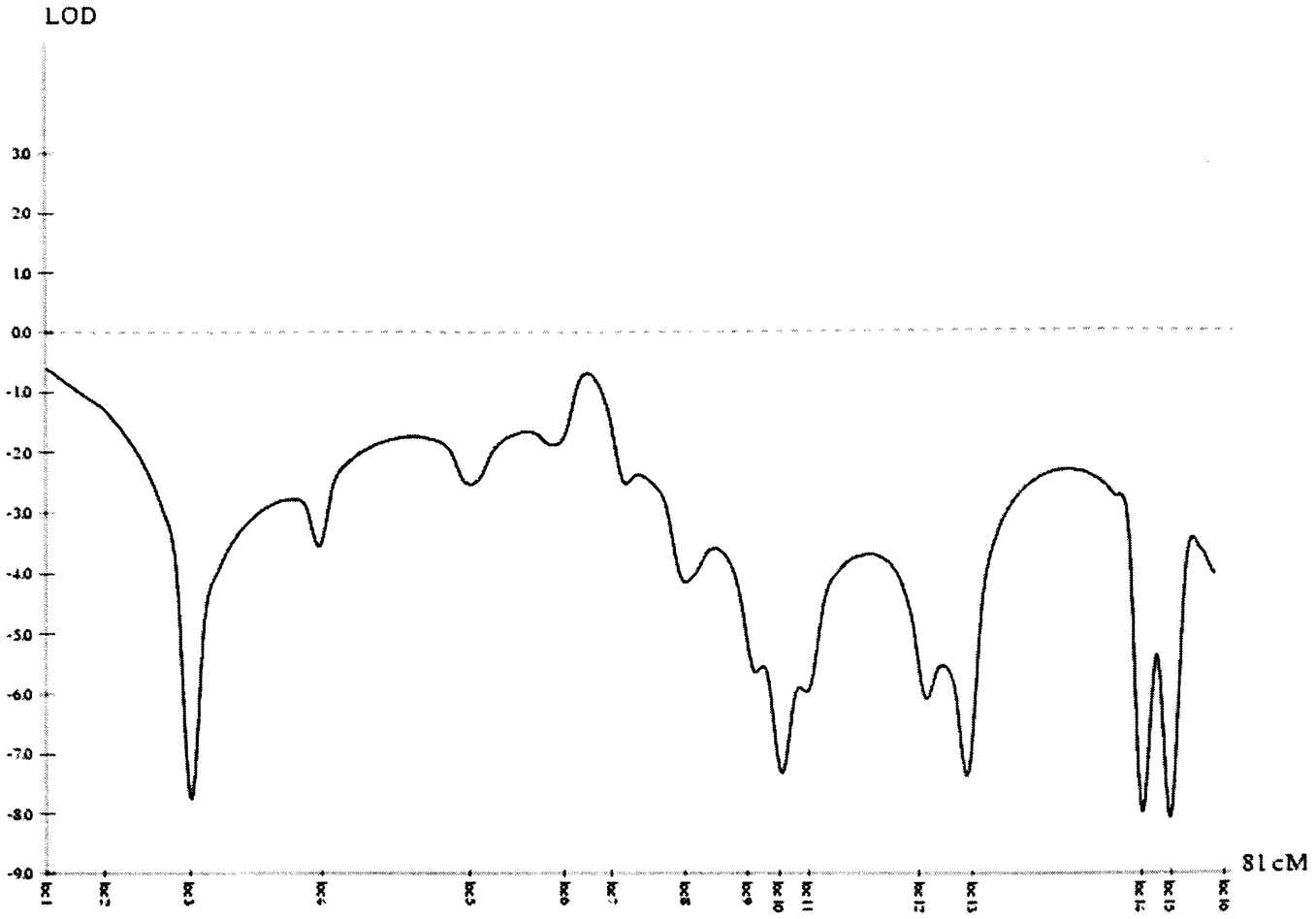


Figure 42. Chromosome 21 multipoint lod score results

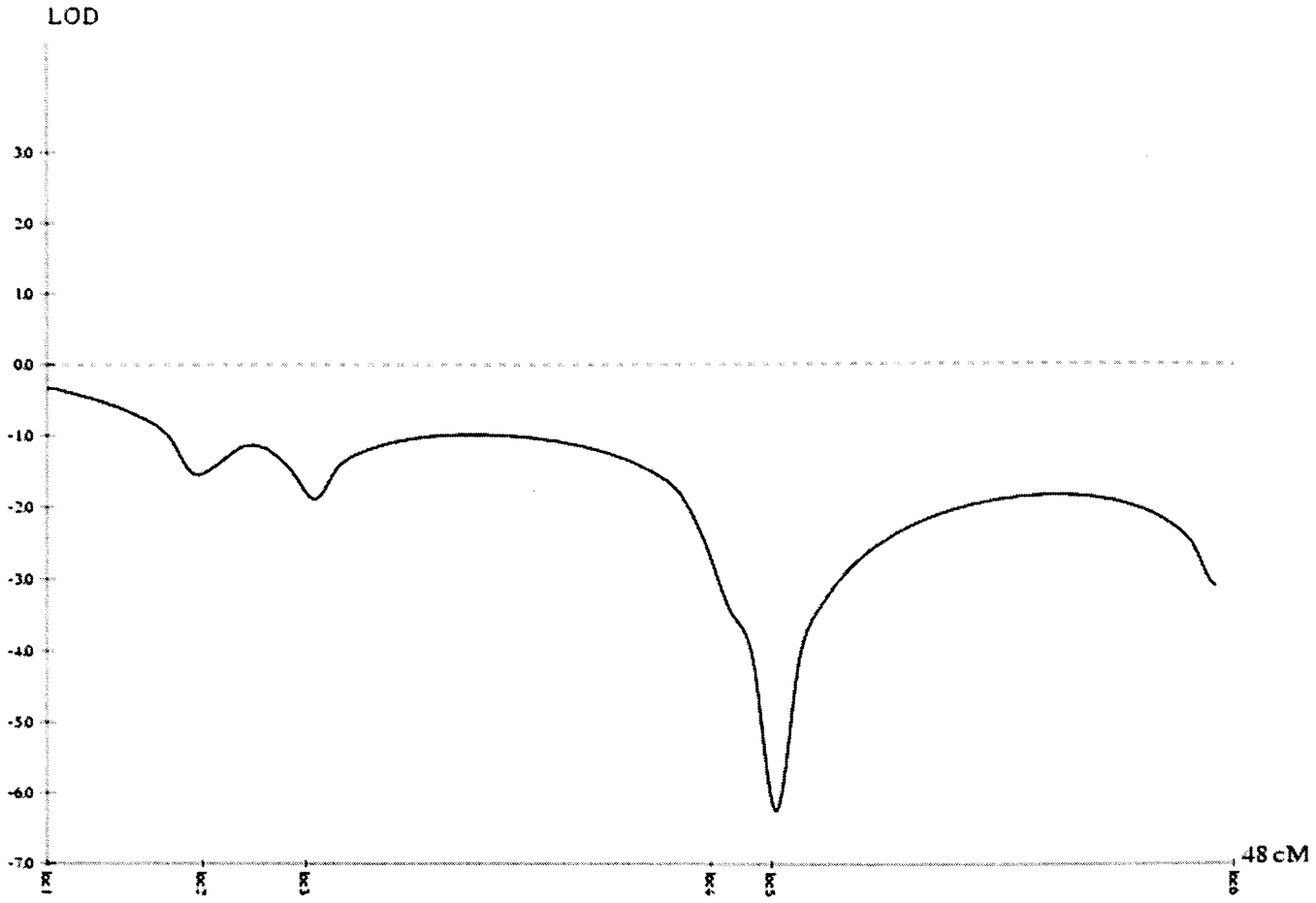
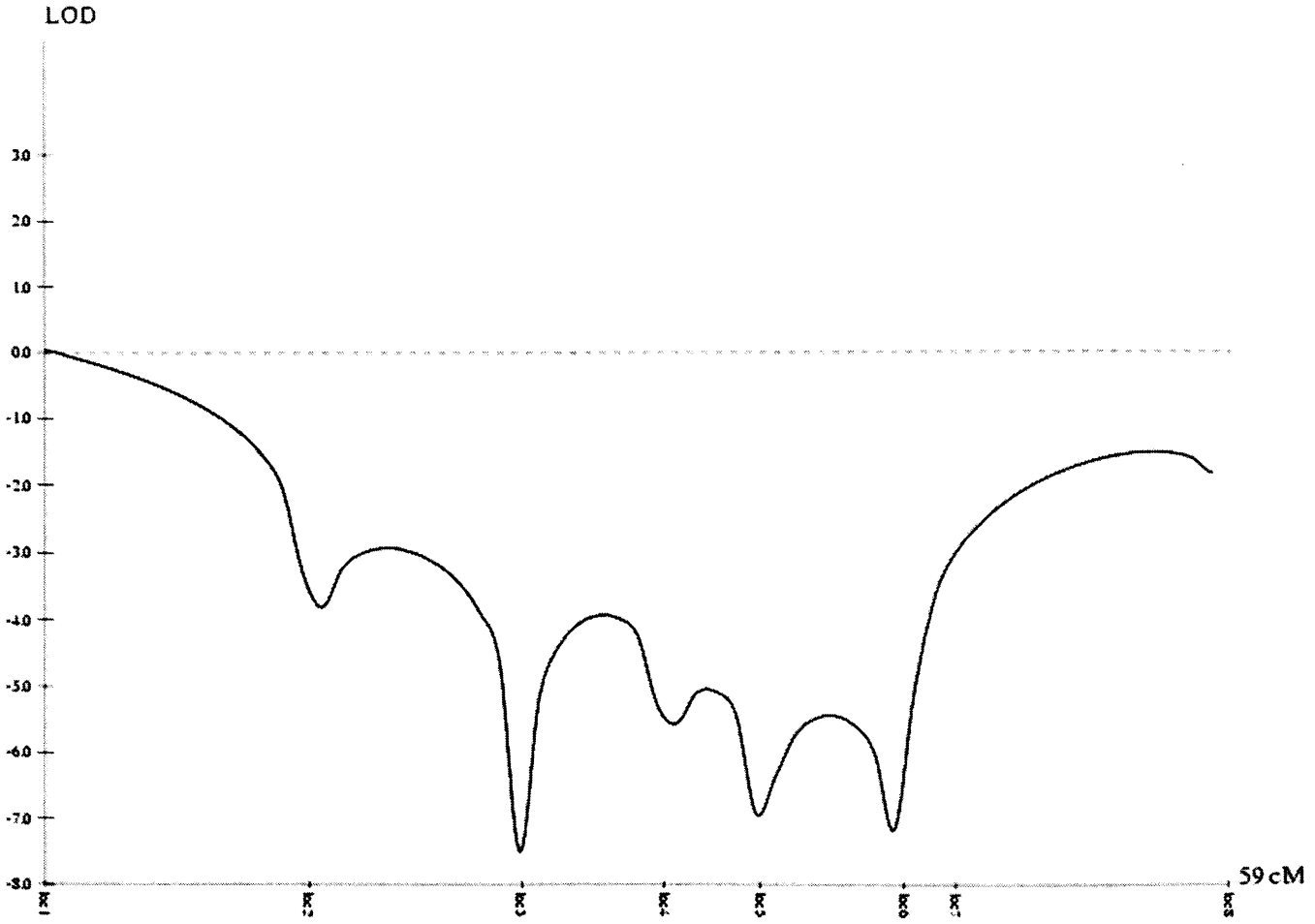


Figure 43. Chromosome 22 multipoint lod score results



5.2.4 Multipoint regions of interest

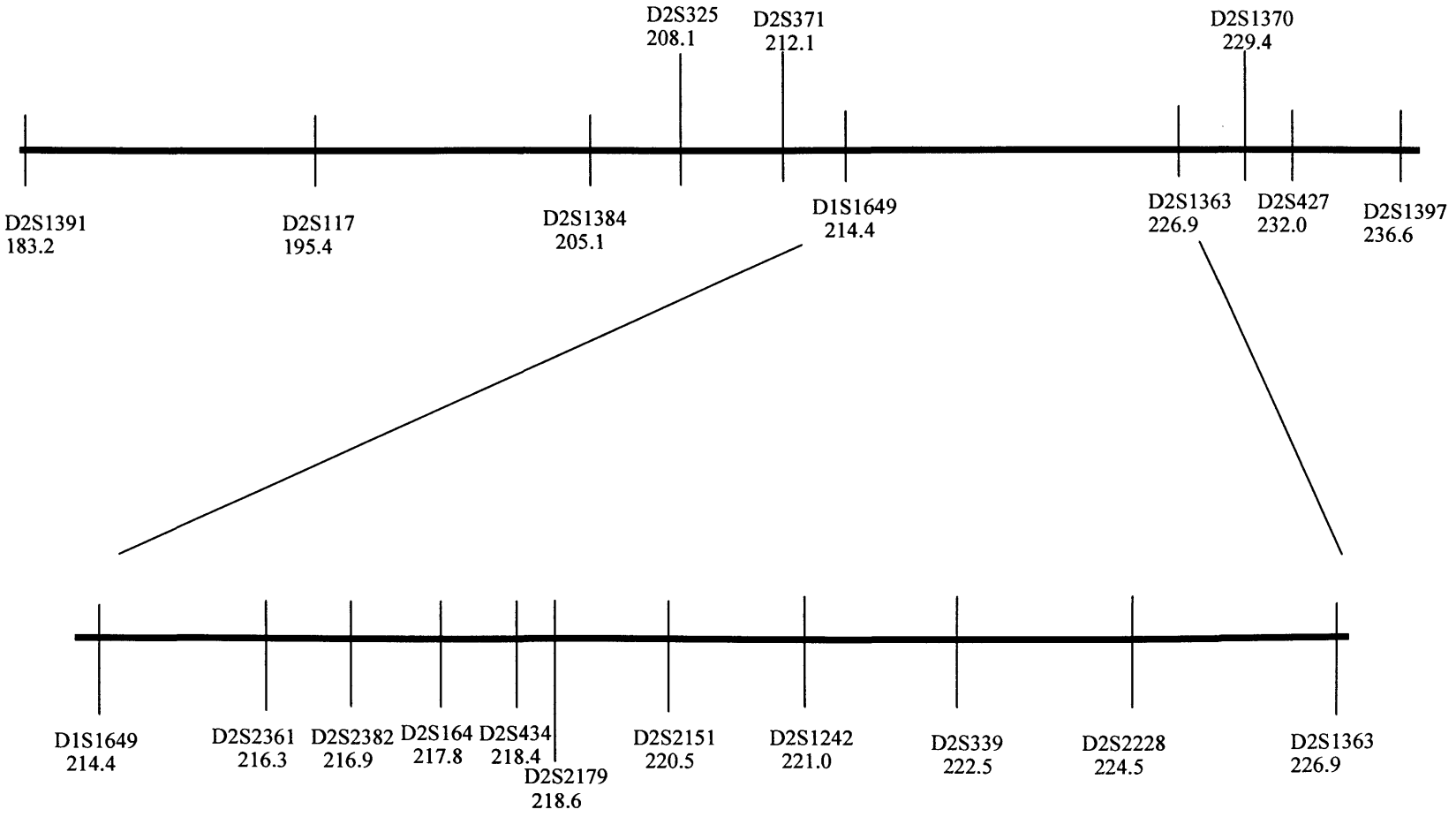
Chromosome 2q32 - 2q37

The highest multipoint lod score across the genome was 2.97 for microsatellite marker D1S1649 on chromosome 2q (the naming of this marker is an anomaly). An adjacent peak was identified for marker D2S1370 with a multipoint lod score of 2.44. Multipoint linkage analysis using information from just the 8 marker loci from the Research Genetics Mapping Set in this region of chromosome 2 gave a maximum two point lod score of 3.03 which is suggestive of linkage.

In view of this result and the possible linkage to this region in a family with an autosomal recessive pattern of inheritance as noted above, the available individuals from our pedigree were genotyped for further markers with a greater density across this area.

The Ensembl database (www.ensembl.org) was searched for markers lying between D1S1649 and D2S1363, and 11 additional markers were selected at an average density of 1.2 Mb apart (figure 44).

Figure 44. Schematic illustration of map locations of microsatellite markers for 2q32 – 2q37. (the distance of each marker along the chromosome is shown in Mb)



The haplotypes for the markers of the Research Genetics Marker Set version 8 within this region of interest have already been discussed (section 5.2.2, figure 15). An examination of the haplotypes of the additional markers studied in this region shows that there two distinct haplotype blocks that are shared by the affected individuals. These haplotype blocks are separated by a distance of 8.2 Mb. There are common alleles in the affected family members successfully genotyped for three adjacent markers, D2S2179, D2S2151 and D2S1242 (figure 45). However, the maximum lod score produced by multipoint linkage analysis across this region was only 0.6 (figure 46). Marker D2S2151 appears to be relatively uninformative in the pedigree, with only two individuals being heterozygous for this marker. The distance spanned by these three markers is 2.2 Mb, and this area potentially overlaps the region of homozygosity identified in the recessive family. The unaffected family member VI:2 also shares the haplotype for these three markers with the affected members.

The affected individuals also share a common haplotype for markers D2S1370 and D2S437 (figure 45). The maximum multipoint lod score in the region of these markers was 1.83 (figure 46). The haplotype for these markers seen in the affected individuals is also present in the unaffected individual VI:2.

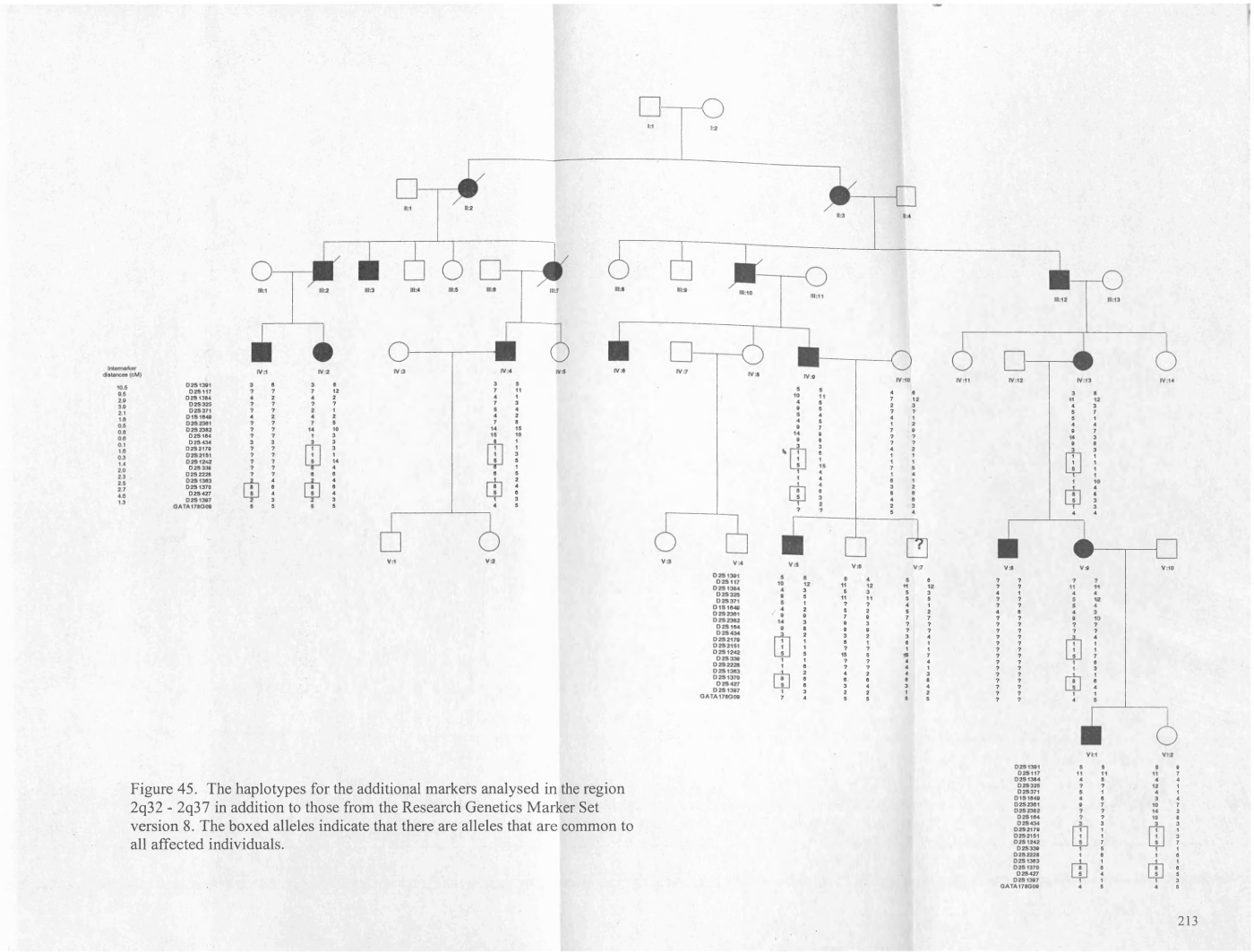
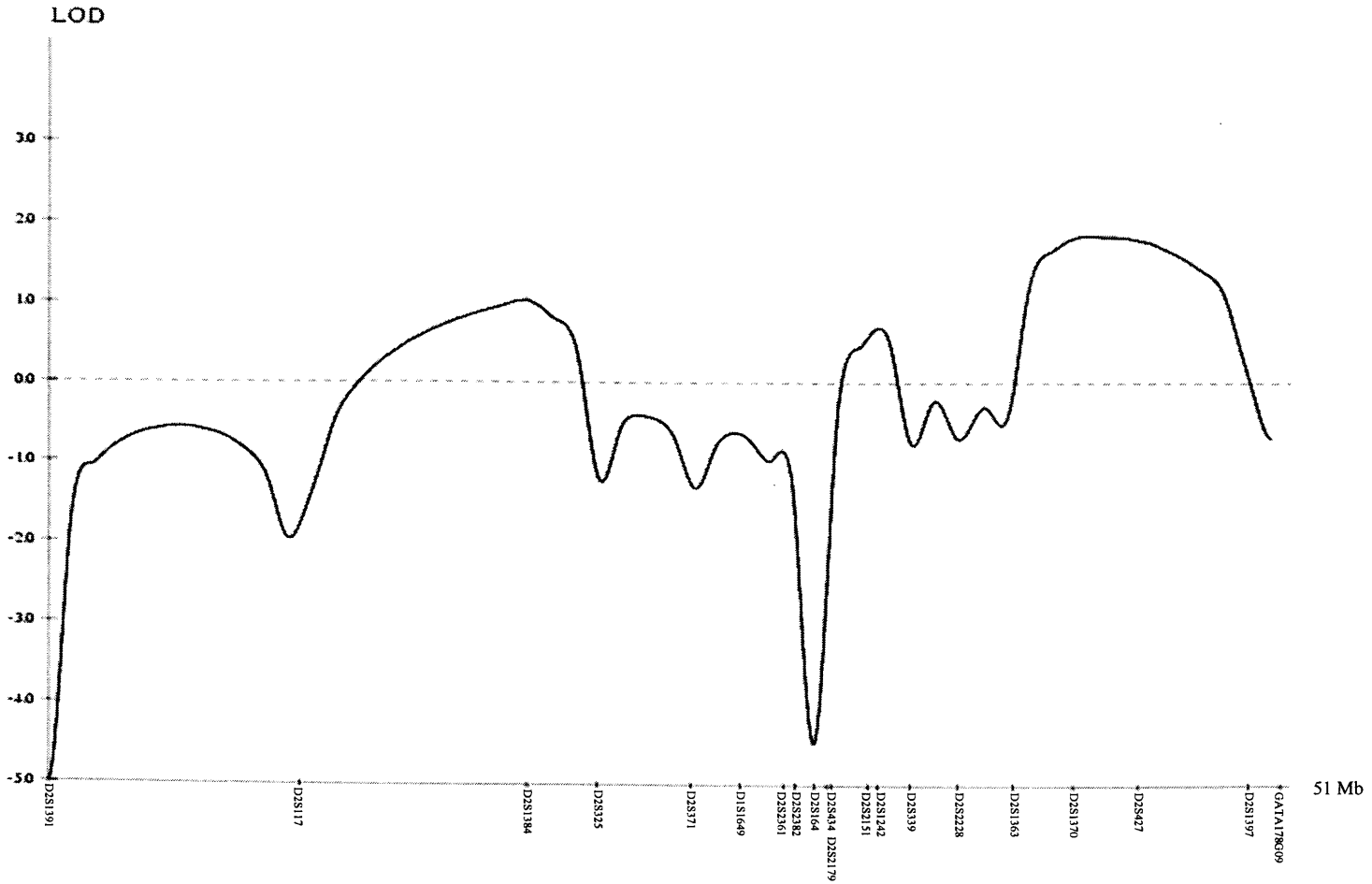


Figure 45. The haplotypes for the additional markers analysed in the region 2q32 - 2q37 in addition to those from the Research Genetics Marker Set version 8. The boxed alleles indicate that there are alleles that are common to all affected individuals.

Figure 46. Plot of the multipoint lod scores for the additional markers analysed in the region 2q32 – 2q37



Chromosome 8p21

The maximum multipoint lod score in this region of 2.52 corresponds to the position of marker D8S1145 (figure 27). Two point lod score analysis for this marker produced a value of 0.68 ($\theta = 0$, GENEHUNTER analysis) (0.69, $\theta = 0$, LINKAGE analysis). All the individuals who were successfully genotyped for this marker shared a common allele, including three subjects who are phenotypically unaffected as shown in figure 47.

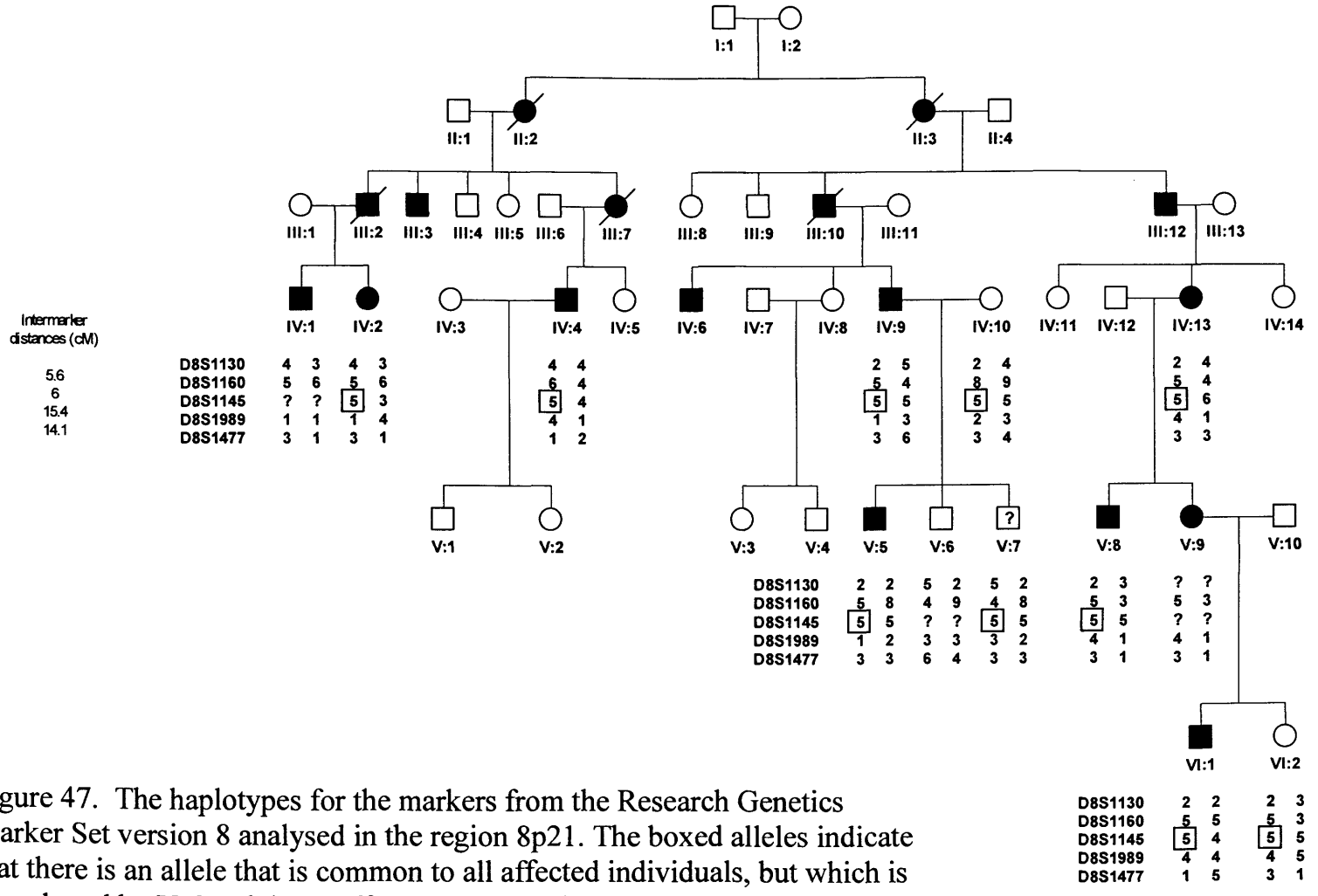


Figure 47. The haplotypes for the markers from the Research Genetics Marker Set version 8 analysed in the region 8p21. The boxed alleles indicate that there is an allele that is common to all affected individuals, but which is also shared by V:7 and the unaffected individuals IV:10, and VI:12.

Chromosome 14q23

Chromosome 14 yielded a maximum lod score of 1.45 at 62cM along the chromosome, which relates to the position of marker D14S588 (figure 35). Only four affected family members were successfully genotyped for this marker, and all were homozygous for allele 2 of this marker (figure 48).

As noted above, analysis of another pedigree with apparent autosomal transmission of hemifacial microsomia has previously mapped the disease gene to 14q32, between markers D14S987 and D14S65 {Kelberman, Tyson, et al 2001 55 /id}. However, that locus is in the telomeric end of the long arm of chromosome 14, and is distinct from the locus with the highest lod score in our genome scan which maps to 14q23, some 26 Mb away.

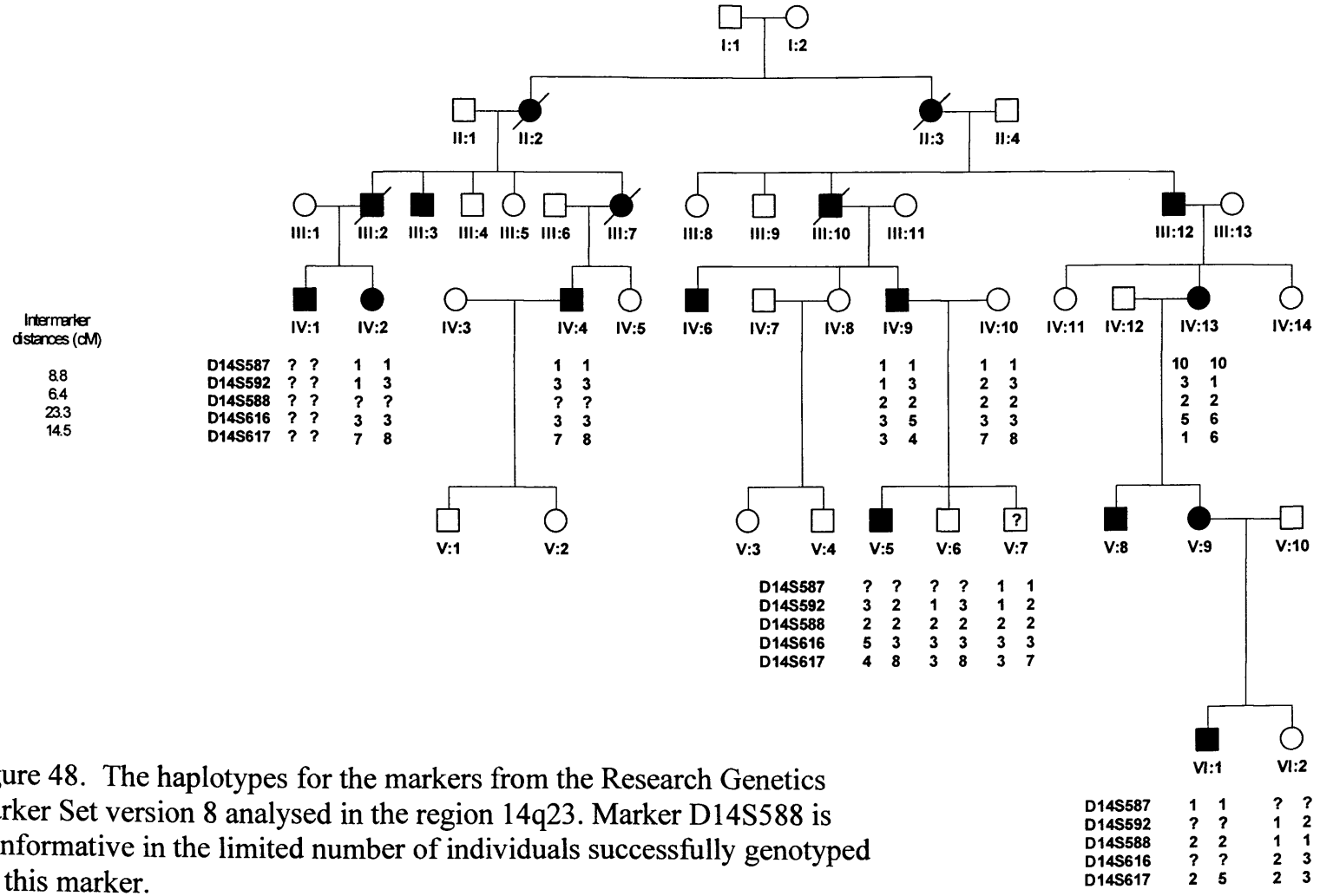


Figure 48. The haplotypes for the markers from the Research Genetics Marker Set version 8 analysed in the region 14q23. Marker D14S588 is uninformative in the limited number of individuals successfully genotyped for this marker.

Chromosome 15q11 - 15q12

Two positive peaks were found on multipoint analysis in this region as seen in figure 36. A lod score of 1.49 was calculated for a point 17 cM from the first marker on chromosome 15, D15S817, and a lod score of 1.07 was calculated a further 19cM along the chromosome. There are no correspondingly high two point lod scores in this area. An examination of the haplotypes, as shown in figure 49, reveals no common haplotype amongst the affected individuals studied.

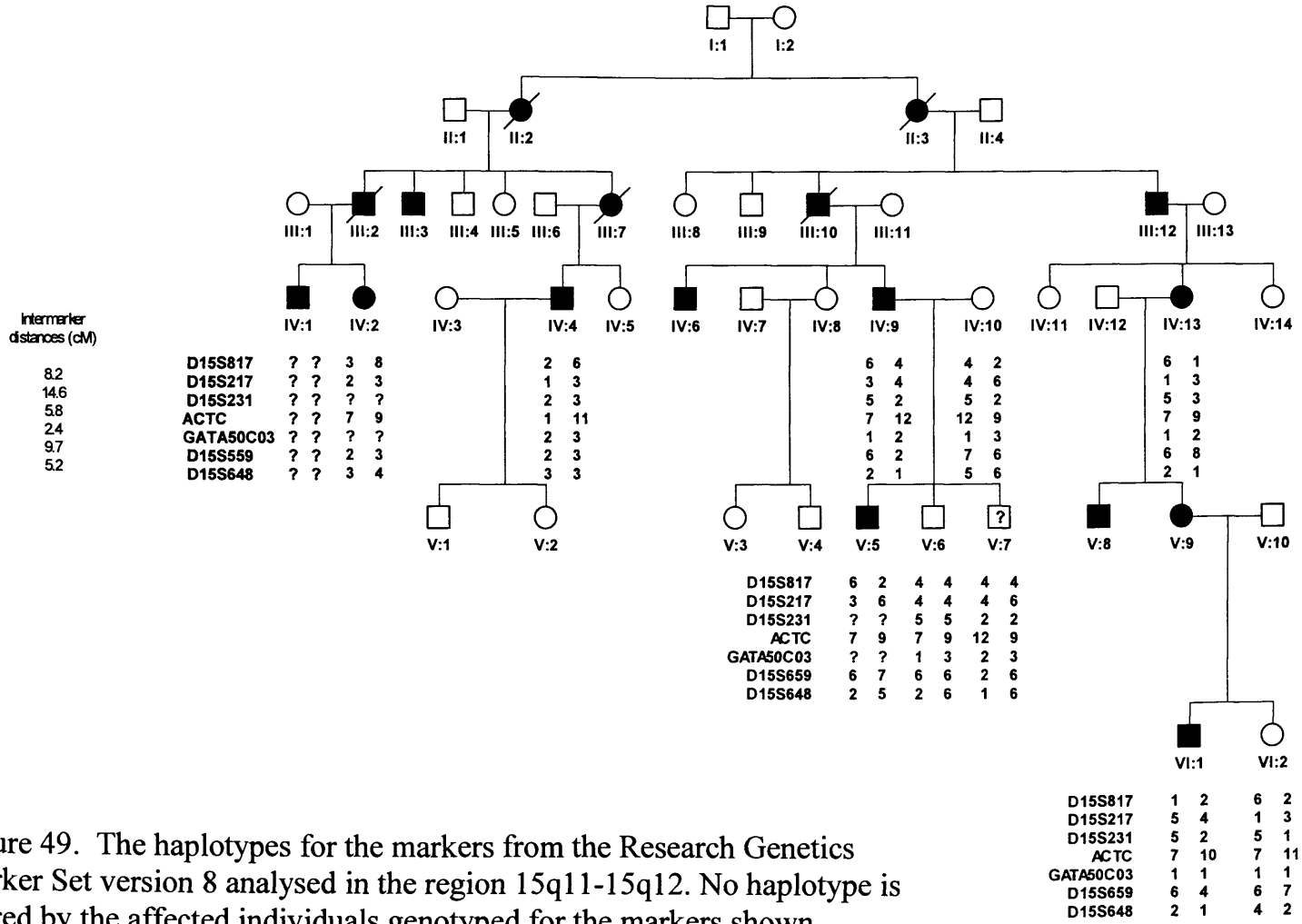


Figure 49. The haplotypes for the markers from the Research Genetics Marker Set version 8 analysed in the region 15q11-15q12. No haplotype is shared by the affected individuals genotyped for the markers shown.

Other regions of potential interest identified by multipoint analysis corresponded to the regions of the markers that had the highest two point lod scores and have been discussed above.

Table 35 Summary of loci for which both the two point and multipoint lod scores were strongly positive

Chromosomal band location	Maximum Multipoint Lod Score using GENEHUNTER analysis (V:7 unknown)	Maximum Two Point Lod Score using GENEHUNTER analysis (V:7 unknown)	Closest Marker analysed to multipoint peak
3q25, 185cM	2.05	2.86	D3S4531
5q34-5q35, 201cM	2.86	3.18	D5S1713
10q22, 94cM	1.58	1.51 ($\theta = 0.1$)	D10S1427
11q25, 140cM	1.57	1.87	D11S2359
13q34 , 101cM	1.60	1.83	D13S895
16p12, 36cM	1.70	1.34	D16S749

6. Discussion of genome scan results

Hemifacial microsomia is one of the most commonly recognised craniofacial malformations. A wide range of defects have been ascribed to this condition, and other terminology such as oculoauriculovertebral spectrum and Goldenhar syndrome are often used synonymously. The rational for considering these varied phenotypes to be manifestations of a single condition is that their characteristic features are all produced by the malformation of structures derived from the first and second branchial arches to be considered.

A number of theories regarding the embryogenesis of hemifacial microsomia have been proposed, including metabolic, teratogenic, vascular and genetic influences, though none of these has been shown to be the primary determinant for the development of the condition. In fact it is most likely that the malformation complexes currently described by the term hemifacial microsomia are actually a conglomerate of aetiologically heterogeneous conditions.

Examination of the pedigrees of affected families reveals a small but significant number of cases in which hemifacial microsomia is inherited in a Mendelian pattern. The observation that 1-2% of cases of hemifacial microsomia have a family history that is suggestive of autosomal dominant inheritance suggests that there is a strong genetic influence in a proportion of cases of hemifacial microsomia. Other evidence pointing towards a major genetic basis include the empirical recurrence risk of 1-2 %, the occurrence of the phenotype in association with a number of chromosomal

rearrangements, and animal models that carry a single gene mutation and that exhibit features reminiscent of those seen in hemifacial microsomia. In order to gain a better understanding of the factors involved in hemifacial microsomia we undertook a genome scan of a family in which the condition appeared to be dominantly segregating, to see if a single major disease associated locus could be identified.

6.1 Genotyping difficulties

The genome scan was performed using the Research Genetics Marker Screening Set version 8.0 which contains primers for 496 microsatellite markers covering the autosomal chromosomes. Blood samples were received from Dr Robert Gorlin from which DNA was extracted by the North East Thames Regional Molecular Genetics Laboratory at Great Ormond Street hospital. Stock solutions of the resultant DNA, at concentrations of 250ng/μl, were aliquoted and further diluted to a concentration of 50ng/μl for use in the PCR amplifications. These 50ng/μl working stocks were stored at 4° C when not in use. The PCR protocol that was followed at the start of the project had been successfully used by co-workers in our laboratory using similar microsatellite markers, and specific bands were observed when the products of the first reactions were examined by agarose gel electrophoresis. However, as the genome scan progressed the absence of a suitable product for various isolated individuals was frequently noted, though there was no obvious pattern with regards to which particular DNA samples failed with the different microsatellite markers. Numerous alterations to the PCR

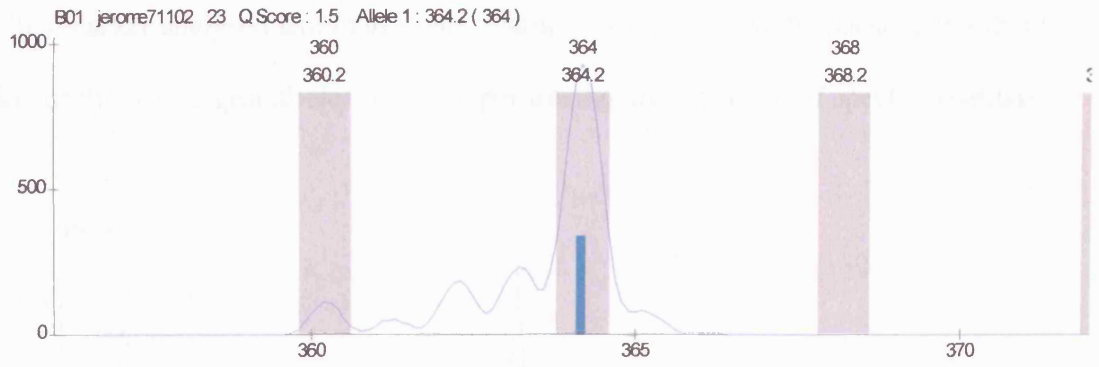
protocol including stepwise reduction of the annealing temperature, increasing the concentration of the primers, and increasing the magnesium concentration were required in order to improve the success of the reactions. Despite all of these measures it was not possible to successfully amplify the study DNA with 46 of the markers from the set, and thus, there are a few regions of the genome which would warrant investigation with alternative markers e.g. chromosome 17q21 – 17q25 that spans 33.9 Mb. The potential reasons for the failures include degradation of the fluorescent labels attached to the primers by repeated exposure to light, suboptimal concentrations of DNA or primers in the reaction mix, or the presence of contaminants.

A secondary deleterious effect of the repeated failure of some of the PCR's was that the limited stock of DNA for each individual was depleted more rapidly than had been foreseen at the start of the genome scan. Further samples were requested from Dr Gorlin, but unfortunately some of these were unobtainable. Thus, 13 pedigree members were genotyped for chromosome 1, but only 10 individuals were typed for chromosome 22.

6.2 Potential sources of error

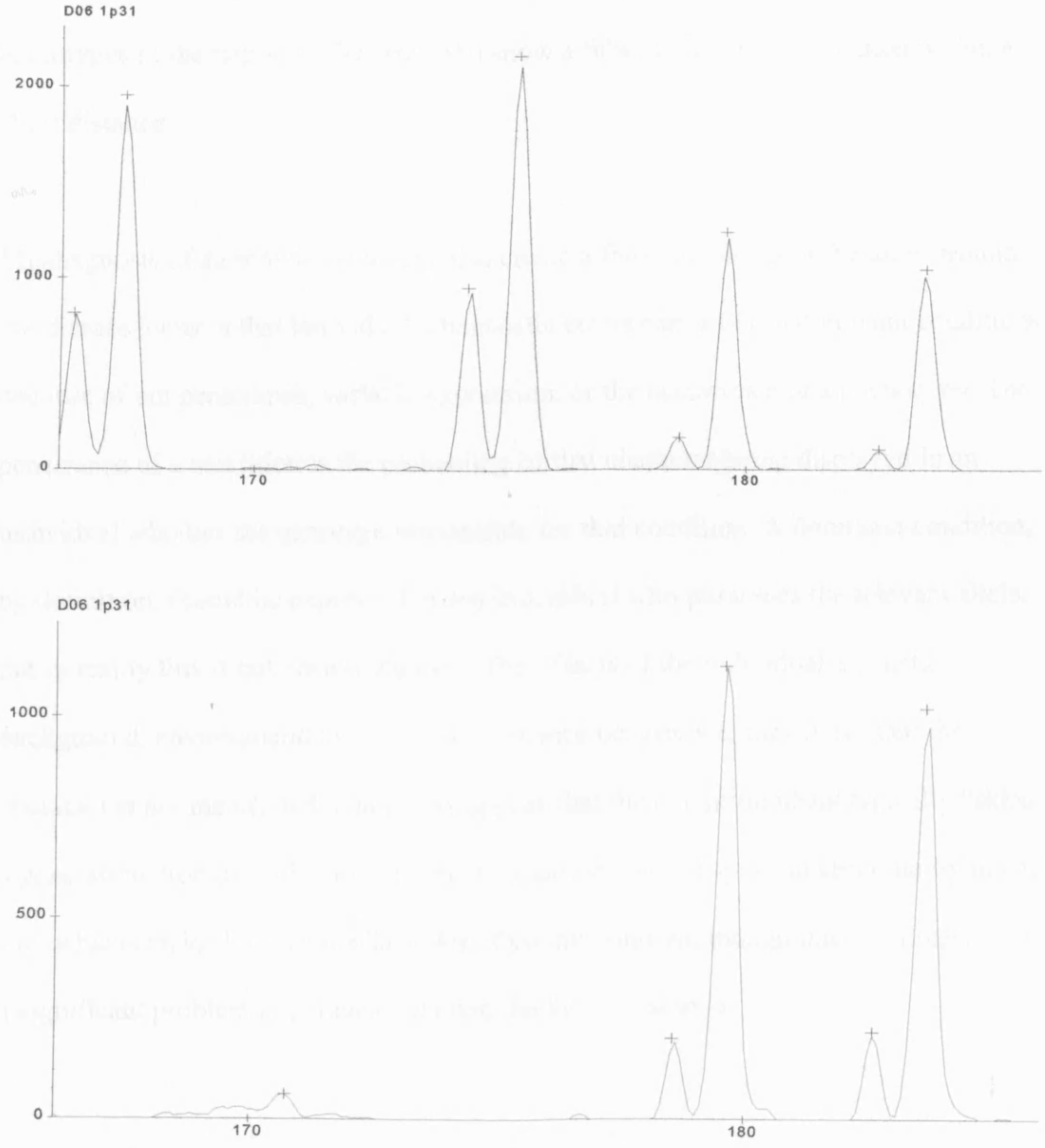
When interpreting the results of any linkage analysis consideration must be taken of the potential sources of error associated with the techniques used. Errors may occur during the initial process of genotyping; sample mislabelling, incorrect allele sizing and assignment, or cross contamination could all lead to the assignment of a false genotype to an individual. Care was taken to ensure that all samples were correctly identified throughout the genotyping and the analysis stages of this study. Polymerase chain amplification of a segment of DNA may produce a population of DNA fragments that includes fragments of a different size to those of the target DNA locus. These additional products tend to differ in size from the original by the size of one or more of the nucleotide repeats in the microsatellite marker used. When sufficient amounts of these unwanted products are formed, they create spurious 'stutter' peaks during the analysis of the genotyping reactions (figure 50). Usually the number of additional fragments is small in comparison to the intended product but occasionally identification of the true size of the original fragment is difficult, though this was rarely the case in this study. The Genetic Profiler software used for genotyping in this study automatically called alleles according to the highest spectrographic peaks found within the size range of the expected products for each marker. However, it was still necessary to review the electropherograms for each marker to ensure that the quality of the trace was reasonable, and that the alleles called were appropriate and complete; any problems relating to stutter were also assessed at the same time.

Figure 50. Output from Genetic Profiler analysis of data showing multiple "stutter" peaks.



A more commonly encountered problem was the automatic calling of false alleles by the Genetic Profiler software because of spectral overlap. The markers of the Research Genetics marker set are labelled with one of three different fluorescent tags, so that the products of three PCR's can be analysed together even if their product sizes overlap. The microsatellites are organised into panels of up to 19 markers that are chosen in such a way that the pooled products of these markers can be analysed together in a single MegaBACE run in order to increase the throughput of samples. However, if there was a very large amount of product for one reaction then spectral overlap sometimes resulted in spurious peaks for the other marker(s) in the panel with the same product size range. This bleed through problem was mitigated by an awareness of its occurrence, and by manual examination of the diagrammatic output for all markers superimposed onto one picture (figure 51). If these measures failed to separate the true from false alleles, the interfering products were reanalysed separately.

Figure 51. Output from Genetic Profiler demonstrating spectral overlap. The upper spectrograph shows four allele peaks in the expected product size range for a single Hex labelled marker. The lower trace reveals two allele peaks relating to a single Tet labelled marker analysed simultaneously. Comparison of the two traces suggests that the peaks for the two largest alleles on the upper tracing are the result of spectral overlap.



If a genotyping error does occur and it results in an offspring being assigned alleles that are not present in its parents' haplotypes then the error may be identified by an examination of the haplotypes in relation to the pedigree. Also, the computer programs used to generate lod scores will stall if the genotypes of the offspring and parents are incompatible. It is possible for genotyping errors to produce a genotype for an individual that is incorrect, but which, theoretically, could have been inherited from their parents. This type of error is more difficult to identify, though examination of the haplotypes in the region of the error will show a false double recombination within a short distance.

Misdiagnosis of an individual would also create a false double recombination around the disease locus in that individual. Diagnostic errors can occur in dominant conditions because of nonpenetrance, variable expression, or the occurrence of a phenocopy. The penetrance of a condition is the probability of that character being displayed in an individual who has the genotype responsible for that condition. A dominant condition, by definition, should be expressed in any individual who possesses the relevant allele, but in reality this is not always the case. The effects of the individual's genetic background, environmental influences, or chance occurrence, may mean that the character is not manifested, and it may appear that the condition phenotypically "skips" a generation. Some dominantly inherited conditions only display an abnormality in adult life, which can lead to the misdiagnosis of young children, though this is unlikely to be a significant problem in patients with hemifacial microsomia.

The affected members of a family bearing a dominantly inherited condition may display different features of the condition, and this is termed variable expression. The explanations for these differences are the same as for non-penetrance, i.e. the effects of other genes, environment and chance. The diagnostic criteria for most conditions are usually defined from observational studies rather than being based upon the features that are known to be produced by the underlying causal mechanism, and this is true for hemifacial microsomia. This may lead to the exclusion of the mildest phenotypes, or the inclusion of causally unlinked conditions that present a similar phenotype. Phenocopies are cases in which the features of the genetic condition of interest are reproduced because of environmental effects, and these are another source of misdiagnosis.

It has been noted in section 1 that the definition and diagnosis of hemifacial microsomia is not straightforward. The literature contains many reports of conditions that are sometimes considered to be synonymous with hemifacial microsomia, e.g. oculoauriculovertebral dysplasia and Goldenhar syndrome, but closer scrutiny of these papers reveals differences in the diagnostic criteria applied. The challenges and complications of misdiagnosis became starkly relevant during the course of this study.

6.3 Change of affection status of individual V:7

During the course of this project the affection status of individual V:7 was called into question (see section 5.2.1). The initial pedigree received from Dr Gorlin indicated that V:7 did not display the hemifacial microsomia phenotype. However, we then learned that he had a pre-auricular skin tag and we assumed that he was affected in the absence of any further information. Direct examination of V:7 some time later revealed that he had had a solitary pre-auricular skin tag with no other features of hemifacial microsomia, and, therefore, his affection status remained debatable. The difficulty in defining the affection status of V:7 arose from the absence of a universally accepted minimum phenotype for hemifacial microsomia, and was compounded because the clinical details of the individuals studied were conveyed to us from another research group. Pre-auricular skin tags or appendages appear to be much more common than hemifacial microsomia or microtia alone. Melnick found skin tags at a rate of 17 per 10 000 amongst a database of over 50 000 pregnancies {Melnick 1980 5 /id}, and Altman reported an even higher incidence of 1.5% {Altman 1951 173 /id}.

For most of the markers there was little difference between the results of the analyses performed with V:7 classed as affected or as affection unknown. There were 8 markers for which, with V:7 classed as affected, the lod score at a recombination fraction of zero excluded linkage, but when V:7 was reclassified as affection unknown the two point lod scores for these markers changed to positive (table 36), and in the case of marker D3S4531 became one of the highest lod scores across the genome scan (table 14). Three

of these eight markers, D2S1384, D1S1649, and D2S427 are within the region of greatest interest, i.e. 2q32 -2q37, given the overall results of the genome scan on family R.

Table 36. Two point lod scores that changed significantly when the affection of individual V:7 was reclassified.

Marker Name	MLINK two point lod score, $\theta = 0$, affecteds only including V:7	GENEHUNTER two point lod score, affecteds only, V:7 affection unknown
D2S1384	-infinity	1.66
D1S1649	-infinity	1.22
D2S427	-infinity	1.13
D3S4531	-infinity	2.86
D5S1456	-2.83	0.79
D8S1985	-infinity	1.68
D10S1213	-infinity	1.04
D15S652	-infinity	1.27

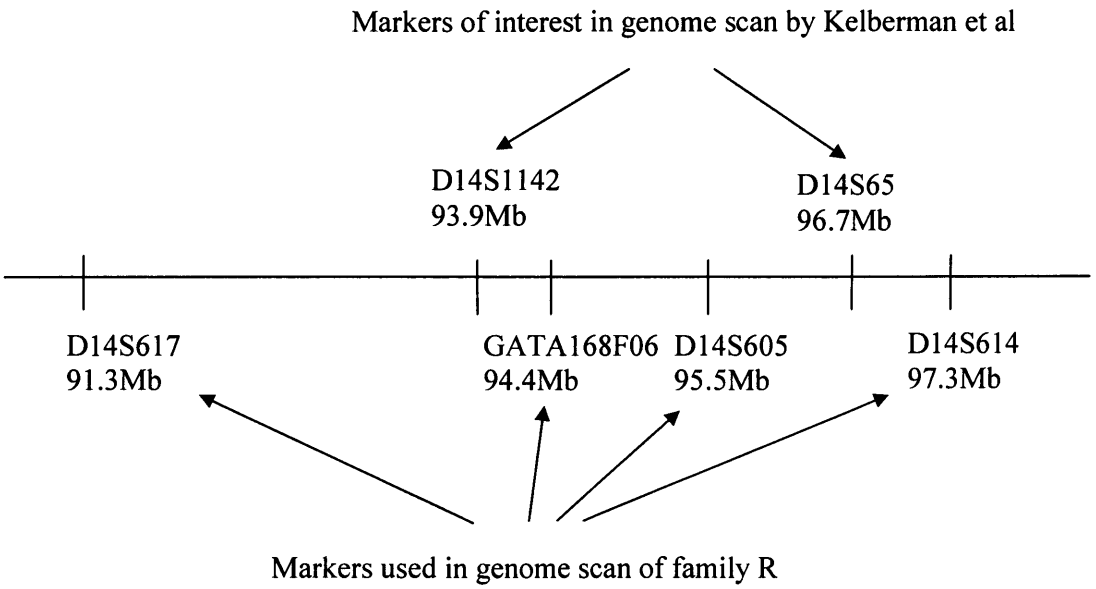
Predictably, when V:7 was reclassified as affection unknown there were no markers for which the two point lod score, at recombination fraction $\Theta=0$, changed from being a positive value to a negative value that would exclude linkage to that marker.

All linkage analyses are reliant on accurate pedigree information, and we were reliant on Dr Gorlin to ensure that the information used was accurate, and that there were no issues with regards to potential problems such as non-paternity.

6.4 Regions of interested excluded by the results of genome scan

A genome wide search has been performed by other authors in another family affected by hemifacial microsomia in which there appears to be an autosomal dominant pattern of inheritance {Kelberman, Tyson, et al 2001 55 /id}. The data from this scan were suggestive of linkage to chromosomal region 14q32. The markers D14S617 and D14S614 used in my genome scan flank the region of chromosome 14q32 that showed potential linkage in the work by Kelberman et al, and two other markers used in my genome scan, GATA168F06 and D14S605, lie within this region of interest as shown in figure 52.

Figure 52. The relative positions of markers in the region 14q32



The highest two point lod score amongst these four markers was 0.94 for GATA168F06 (GENEHUNTER analysis, $\theta = 0$); the other three markers returned two point lod scores that were more negative than –2 at a recombination fraction of zero. The highest multipoint lod score across the region of these four markers was –0.6, which again corresponded to the position of GATA168F06; it seems unlikely that the disease gene in family R is located between these markers, though the results do not definitively exclude linkage to the whole of this region.

Graham et al {Graham, Hixon, et al 1995 80 /id} presented a brief report of another family showing autosomal dominant transmission of a Goldenhar-like syndrome, and showed linkage to the same region of chromosome 8 to which branchio-oto-renal syndrome maps. Markers D8S1113, D8S1136 and D8S2323 lie within this region of interest, 8q11-8q13. Markers D8S1113 and D8S2323 yielded two point lod scores of less than –2, and D8S1136 a two point lod score of –1.93 in the GENEHUNTER analysis of affecteds only (table 19). The multipoint lod scores are also negative across this region, and therefore, linkage to this region appears most unlikely.

It has been noted in section 1.6.4 that the hemifacial microsomia phenotype has been associated with a number of chromosomal rearrangements. Hemifacial microsomia with deletion of 6q has been reported {Greenberg, Herman, et al 1987 141 /id}, but linkage to this region in family R seems unlikely as none of the multipoint lod scores calculated for 6q was positive.

Trisomy 7 and duplication of 7q have been noted in separate reports of hemifacial microsomia {Hodes, Gleiser, et al 1981 95 /id} {Hoo, Lorenz, et al 1982 96 /id}, but in the study of family R all the two point lod scores for this chromosome calculated using only the affected individuals are below –2, and the highest multipoint lod score was only 0.41.

Similarly, there are published reports of trisomy 9 mosaicism and hemifacial microsomia {Wilson & Barr 1983 42 /id} {de Ravel 2001 162 /id}, but the two point lod scores from our genome scan are almost all strongly negative for chromosome 9, and apart from the region between the first two markers the multipoint lod scores are all negative as well.

The results of our genome scan do not suggest linkage to chromosome 18, several rearrangements of which have been reported in association with hemifacial microsomia {Bersu & Ramirez-Castro 1977 97 /id} {Greenberg 1987 141 /id} {Verloes 1991 165 /id} {Clarren & Salk 1983 98 /id} {Sujansky & Smith 1981 110 /id}. For chromosome 18 all but one of each set of two point lod scores (MLINK including all individuals, MLINK affecteds only, and GENEHUNTER) were negative, and all the multipoint lod scores calculated were negative as well.

The multipoint lod scores for chromosome 22 exclude linkage to most of this chromosome, and all scores were negative. This chromosome was of interest because of a number of case reports {Greenberg 1987 141 /id } {Herman, Greenberg, et al 1988 68

/id} {Kobrynski, Chitayat, et al 1993 70 /id} {Pridjian 1995 171 /id} {Hathout, Elmendorf, et al 1998 69 /id} {de Ravel 2001 162 /id}.

6.5 Lod scores relative to candidate genes suggested by animal models

There are several candidate genes for hemifacial microsomia that have been suggested as a result of animal models (see section 1.6.10). Most of these genes are in close proximity to markers used in the genome scan, and the results are summarised below (table 37). Only *FGF8* would appear to merit further consideration since it is located close to marker D10S1239 that was associated with a multipoint lod score of 1.47. *FGF8* is one of the fibroblast growth factors, which are a group of proteins that regulate growth and development. *Fgf8* has been shown to be required for left right axis determination in both chick and mouse studies {Meyers & Martin 1999 7 /id}. Trumpp et al {Trumpp, Depew, et al 1999 119 /id} inactivated *Fgf8* in mice and showed that the gene was required for cell survival and patterning of the first branchial arch. It is also known that *FGF8* regulates the expression of other potential hemifacial microsomia disease genes, having been implicated in HOX gene activation {Dubrulle, McGrew, et al 2001 191 /id}, and regulation of *DLX-1* and *DLX-2* {Shigetani, Nobusada, et al 2000 190 /id} {Thomas, Liu, et al 2000 133 /id}.

The candidate gene *GSC* lies with the region 14q32 that has been discussed above. *GSC* lies 94.3Mb along chromosome 14 just proximal to marker GATA168F06, the results for which do not encourage further investigation at this stage.

Table 37. Maximum lod score results in relation to candidate genes suggested by animal models of hemifacial microsomia

The candidate gene and its position along the chromosome is shown in the central shaded column, together with the markers from the Research Genetics Marker Set that are located on either side of the gene locus. The maximum lod score from all of the three two point analyses for each of the markers is shown, together with the maximum multipoint lod score in each region.

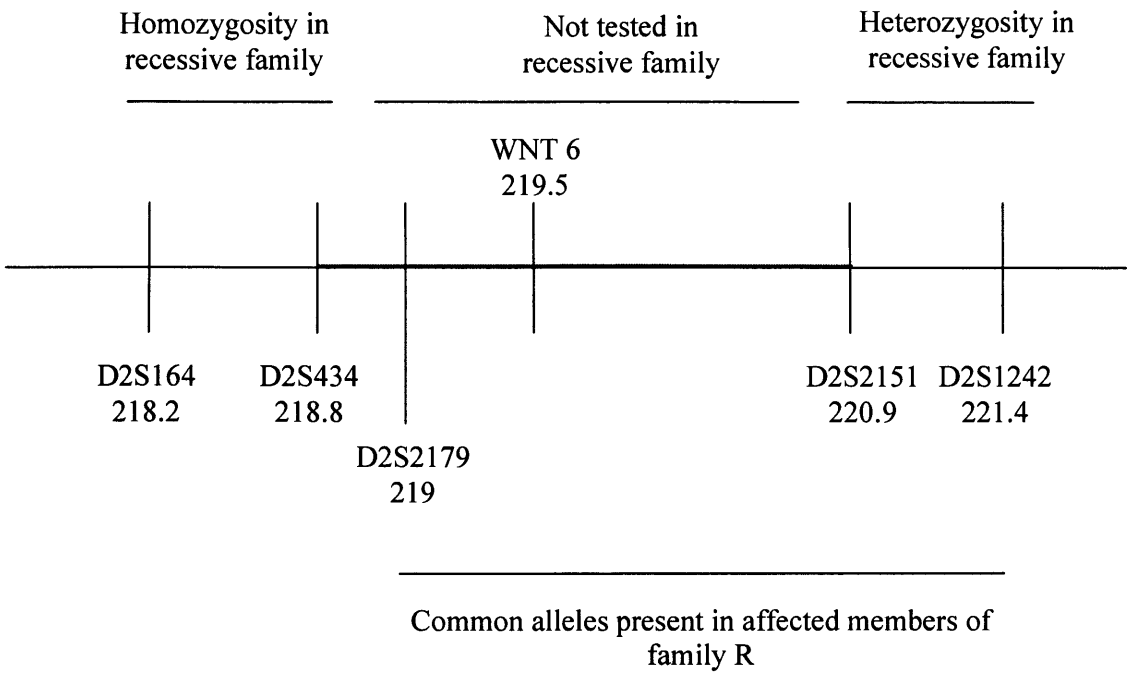
Chromosomal region	Marker name	Maximum two point lod score	**CANDIDATE GENE**	Marker name	Maximum two point lod score	Maximum multipoint lod score
2q31	D2S1776 169.5Mb	0.01 (MLINK, all individuals, $\theta = 0.4$)	*DLX-2* 172.3Mb	GATA194A05 176Mb	0.5 (MLINK, all individuals, $\theta = 0.4$)	-0.82
2q31	D2S1776 169.5Mb	0.01(MLINK, all individuals, $\theta = 0.4$)	*DLX-1* 172.8Mb	GATA194A05 176Mb	0.5 (MLINK, all individuals, $\theta = 0.4$)	-0.82
3p24	D3S3038 21.9Mb	-0.01 (MLINK, all individuals, $\theta = 0.4$)	*RAR-β* 25.3Mb	D3S2466 26Mb	-0.03 (MLINK, all individuals, $\theta = 0.4$)	-2.65
4q32.2	D4S1625 143.9Mb	0.03(MLINK, all individuals, $\theta = 0.4$)	*EDNRA* 149Mb	D4S1629 158.7Mb	-0.01(MLINK, all individuals, $\theta = 0.4$)	-3.38
4q34	D4S2368 169.1Mb	0.01(MLINK, all individuals, $\theta = 0.4$)	*DHAND* 174.8Mb	D4S2431 175.2Mb	-0.1 (MLINK, all individuals, $\theta = 0.4$)	-4.33

Chromosomal region	Marker name	Maximum two point lod score	**CANDIDATE GENE**	Marker name	Maximum two point lod score	Maximum multipoint lod score
6p24	D6S1955 10.3Mb	-0.02 (MLINK, affecteds only, $\theta = 0.4$)	*AP-2α* 10.5Mb	D6S1279 12.3Mb	0.11 (MLINK, affecteds only, $\theta = 0.3$)	-1.83
6p24	D6S1034 12.3Mb	0.02 (MLINK, affecteds only, $\theta = 0.4$)	*EDN-1* 12.4Mb	D6S1266 19.1Mb	0.08 (MLINK, affecteds only, $\theta = 0.3$)	-1.53
7p15.2	GATA124D01 21.0Mb	0.36(MLINK, affecteds only, $\theta = 0.2$)	*HOXA1* 26.9Mb	D7S1808 27.8Mb	-0.14(MLINK, all individuals, $\theta = 0.4$)	-2.00
10q24	D10S1239 103.2Mb	0.06 (GENEHUNTER, $\Theta = 0$)	*FGF8* 103.5Mb	D10S1246 110.9Mb	0.5 (MLINK, all individuals, $\theta = 0.1$)	1.47
12q13	D12S398 51.5Mb	0.57 (MLINK, affecteds only, $\theta = 0$)	*RAR-γ1* 51.9Mb	D12S1056 58.8Mb	0.01 (MLINK, affecteds only, $\theta = 0.4$)	-1.82
17q21	D17S1294 25.4Mb	0.08 (MLINK, all individuals, $\theta = 0.3$)	*RAR-α* 35.8Mb	D17S1299 36.3Mb	0.28 (MLINK, all individuals, $\theta = 0.2$)	-0.57
17q21.3	D17S1299 36.2Mb	0.28(MLINK, all individuals, $\theta = 0.2$)	*HOXB1* 47.1Mb	D17S968 70.1Mb	0.1(MLINK, all individuals, $\theta = 0.4$)	0.18

6.6 Regions of interest warranting further investigation

The genome scan of family R produced a number of regions in which the maximum two point and multipoint lod scores were both positive, and it is amongst these regions that the disease locus is most likely to be identified. Chromosome 2q32 – 2q37 produced the highest multipoint lod score in the whole genome scan, and it appeared from the lod plot (figure 22) that there were two contiguous areas, 40cM and 23cM in size, across in which the multipoint lod scores were strongly positive. This finding gained added significance because previous work had identified this area as a region of homozygosity in a family with recessively inherited hemifacial microsomia (personal communication M Bitner-Glindzicz and J.Tyson), and hence the decision was taken to examine further microsatellite markers in this region in the limited time available at the end of this project. An examination of the haplotypes for these additional markers suggests that there are two distinct areas within this region within which there is allele sharing amongst the affected individuals who were genotyped. However, the maximum multipoint lod score generated by the data from these additional markers was only 0.6 (figure 46). Therefore, it seems less likely that this is the disease gene locus, although there is still an area of interest covered by these markers that overlaps the previously described homozygosity region as shown in figure 53.

Figure 53. Schematic illustration of map locations of microsatellite markers for 2q32 – 2q37. (distances along chromosome in Mb). There is a potential overlap of the regions of interest between markers D2S434 and D2S151.



A search of the ensemble database (www.ensembl.org) for known genes in this region revealed *WNT6* to be the most likely candidate gene. The WNT genes are a family of highly conserved developmental control genes, and *WNT6* has been shown to be expressed in the ectoderm overlying the pharyngeal arches in chick, and is also expressed in the inner ear.

Marker D3S4531 located in the region 3q25 yielded a two point lod score of 2.86 and a multipoint lod score of 2.05 (GENEHUNTER analyses). A review of the expression patterns and functions of known genes in the vicinity of this marker identified *SHOX2* as a possible candidate gene. *SHOX2* is a homeobox gene that has been identified through its homology with the murine gene *Og-12*. *Og-12* is highly expressed in craniofacial tissues including the ear and palate, as well as in the heart, brain and developing limbs {Blaschke, Monaghan, et al 1998 23 /id}. *USH3A* also lies within this region, though this would seem an unlikely candidate for hemifacial microsomia since mutations have been associated with Usher syndrome type III, that is characterised by sensorineural deafness, variable vestibular dysfunction, and retinitis pigmentosa {Ahmed, Riazuddin, et al 2003 9 /id}.

There are two reports in the literature of hemifacial microsomia with cri-du chat syndrome caused by deletion of chromosome 5p {Neu, Friedman, et al 1982 66 /id} {Ladekarl 1968 94 /id}. The critical chromosomal region involved in cri-du-chat syndrome has been identified as 5p12.2- 5p12.3 {Mainardi, Perfumo, et al 2001 130 /id}. The telomeric region of 5p was also involved in an unbalanced translocation with the short arm of chromosome 8 reported in two siblings with hemifacial microsomia {Josifova, Patton, et al 2004 172 /id}. The translocation in these cases resulted in monosomy for 5p15.31 to 5pter and trisomy for the p23.2 to 8pter regions. The most positive result from our two point linkage analyses for the short arm of chromosome 5 was for marker D5S1470, which maps to 5p14, that yielded two point lod scores of 2.17 (MLINK analysis, $\theta = 0$) and 1.87 (GENEHUNTER analysis, $\theta = 0$). There is a shared

haplotype amongst the affected members of family R who were genotyped for this marker and for the adjacent marker GATA145D09. The multipoint lod scores exclude linkage to most of 5p, including the region 5p15.31 to 5pter, and the critical region for cri-du-chat syndrome, although there is a small positive peak in the region of marker D5S1470 with a maximum score of 0.45. A review of the expression patterns and functions of known genes in this region did not reveal any obvious candidates for a hemifacial microsomia disease gene.

Chromosome 5q34-5q35 contained the highest two point lod score in the genome scan of family R of 3.18 ($\theta = 0$, GENEHUNTER analysis) for marker D5S1471, and yielded a maximum multipoint score of 2.86. However, it has been noted in section 5 that there is no haplotype that is identical by descent and exclusive to all the affected individuals studied. Potential candidate genes in this region include *FOXI1*, a member of the forkhead family of winged helix transcription regulators, which are important in the embryogenesis of mammals. Targeted disruption of this gene in mice causes signs of vestibular dysfunction and hearing impairment {Hulander, Wurst, et al 1998 131 /id}. Another gene of interest is *MSX1*, which is a homeobox gene involved in craniofacial skeletal formation. *MSX1* is expressed in areas of cephalic neural crest cell migration, and mice deficient for the gene exhibit craniofacial malformations including cleft lip and palate, mandibular hypoplasia, and abnormalities of the malleus. {Satokata & Maas 1994 103 /id} {Blin-Wakkach, Lezot, et al 2001 104 /id}.

The multipoint lod scores for chromosomal region 8p21 – 8p23 are positive over quite a wide 25cM region, with a maximum multipoint lod score of 2.52. *FGF17* is located in the region 8p21, and the members of the FGF family are involved in patterning, cell proliferation and differentiation during embryogenesis. *FGF17* is broadly expressed in the midfacial ectoderm in early embryogenesis {Bachler & Neubuser 2001 132 /id}.

The multipoint lod scores for a relatively large section, 25cM, of chromosome 10 were positive, with a maximum score of 1.58. The chromosomal region is large because of the failure of amplification of three markers in this area. Fibroblast growth factor 8, *FGF8*, maps to10q24.3, and has been discussed as a candidate gene in section 1.6.10. *Fgf8* is involved in left-right axis determination in the mouse {Meyers & Martin 1999 7 /id},with *Fgf8* being a left determinant. *Fgf8* also appears to have an important role in brain, limb and tooth development. Thomas et al {Thomas, Liu, et al 2000 133 /id} found that *Fgf8* is expressed in the epithelium overlying the first branchial arch and regulates the mesenchymal expression of *Dlx2* in mice.

Retinoids are regulators of cell proliferation, differentiation and morphogenesis during embryonic development. CYP26 family members, such as *CYP26C1* that maps to chromosome 10q23, metabolise retinoic acid and are involved in the control of retinoic acid levels in tissues and cells. Murine *Cyp26c1* has been shown to be expressed in prospective rhombomeres 2 and 4, in the first branchial arch and along the mesenchyme adjacent to the rostral hindbrain in the early developmental stages (E8.0 – E8.5) {Tahayato, Dolle, et al 2003 134 /id}. *Cyp26c1* expression persists in rhombomere 2

and in the maxillary and mandibular components of the first branchial arch at E9.5. It is of interest that the hemifacial microsomia phenotype has been associated with the exposure of pregnant women to retinoic acid {Lammer, Chen, et al 1985 77 /id}. Another member of the retinoic acid metabolising cytochrome p450 family, *Cyp26a1*, is also located in this region of interest of chromosome 10, and expression of *Cyp26A1* has been found in the neural-crest derived mesenchyme of the branchial arches in early embryonic mice {MacLean, Abu-Abed, et al 2001 135 /id}.

Another candidate gene in this region is *CHUK* (*Ikk1, IkkA*). *Ikka*$^{-/-}$ mice died within 4 hours of birth and had abnormal limb and craniofacial development {Takeda, Takeuchi, et al 1999 136 /id}. Hu et al {Hu, Baud, et al 1999 137 /id} also generated *Ikka* deficient mice; at embryonic day 18.5 these mice had truncated snouts and no external ears, limbs represented by rudimentary protrusions, and heads that were shorter than normal. They also had fused sacral and cervical vertebrae, a short sternum, an omphalocele, taut skin and they lacked well formed tails. Sil et al {Sil, Maeda, et al 2004 138 /id} found that *Ikka* affects the expression of fibroblast growth factor (FGF) family members, and the expression of another candidate gene, *Fgf8*, is elevated in the limb bud ectoderm of *Ikka* deficient mice.

The homeobox gene *NKX2C* is located at 10q24.2. This gene is expressed in the pharyngeal floor and pouches, as well as in oral, first, and possibly second, branchial arch ectoderm {Biben, Wang, et al 2002 139 /id}. *Nkx2c* deficient mice were born with

gut and spleen abnormalities {Pabst, Zweigerdt, et al 1999 142 /id}, and Biben et al found that *Nkx2c* null mice had abnormal sublingual glands and molar teeth.

Marker D11S2019 from the Research Genetics marker set lies within the region 11q12-13 that has been proposed as a potential hemifacial microsomia disease locus {Singer, Haan, et al 1994 53 /id}, but unfortunately this was one of the markers that did not yield a satisfactory product despite repeated attempts at PCR amplification.

The homeobox gene *BARX2* is located in the region of interest 11q25 that yielded a two point lod score of 1.87 and a multipoint lod score of 1.57. Jones et al {Jones, Kioussi, et al 1997 143 /id} observed expression of *Barx2* in the ectodermal lining of the mandibular and maxillary processes during craniofacial development in the mouse. Intense expression of *Barx2* was observed in small groups of cells undergoing tissue remodelling including ectodermal cells in indentations around the eye and maxillo-nasal groove, and in the first branchial pouch, lung buds, precartilagenous condensations and mesenchyme of the limb. Jones et al suggested that *BARX2* may control the expression of neural cell adhesion molecules, including L1-CAM, and other target genes during embryonic development. Smith and Tabin {Smith & Tabin 1999 144 /id} isolated the chicken gene ***cbarx2***, and noted its expression in craniofacial structures, regions of the neural tube, and muscles in the limb, neck and cloaca.

Region 13q34 yielded a maximum two point lod score of 2.82 and a multipoint lod score of 1.83. Within this region lies *CUL-4A* that appears to have an essential function for early embryonic mammalian development, since homozygous and heterozygous mouse mutants for a *Cul-4a* deletion died during gestation {Li, Ruiz, et al 2002 145 /id}.

Chromosomal region 16p12 was associated with a maximum two point lod score of 1.34, and a multipoint lod score of 1.70. A candidate gene within this region is *COG7* that is involved in the determination of Golgi apparatus structure. Two siblings with homozygous mutations of *COG7* have been reported whose phenotype included low-set dysplastic ears, micrognathia, short neck and loose, wrinkled skin {Wu, Steet, et al 2004 146 /id}.

Thus, there are a number of regions that have been identified from the results of the genome scan as warranting further investigation, and region 2q32-37 would appear to be the most promising at present. The fact that the scan failed to produce a single particular locus that was strongly suggestive of linkage was disappointing. Amongst the reasons for this failure may have been the fact that some of the attempted PCR amplifications failed, and so some areas of the genome were more sparsely covered that initially intended. It has been noted above that towards the end of the study some of the DNA samples ran out, so that, whilst linkage analysis was still possible, the magnitude of the lod scores calculated may have been reduced. The size of family R and the

availability of DNA from only selected members of the pedigree may also have precluded the possibility of a greater lod score.

It is also possible that the genetic defect in hemifacial microsomia is a small deletion, and this would be missed by the genome scan if none of the markers analysed fell within the deletion. A deletion that included a single marker locus might also be overlooked; the affected individual would be typed as a homozygote for the deleted marker assuming that the parental alleles were compatible. A small deletion might be detected by higher resolution analysis of DNA copy number variations. Gene dosage variations have been studied using comparative genomic hybridisation, a technique that utilises differentially labelled total genomic DNA from test and reference cell populations. This DNA is hybridised to either normal metaphase chromosomes, or, more recently, to arrays of mapped sequences. The resulting ratio of fluorescence intensities at a locus is proportional to the ratio of copy numbers of the test and normal DNA at that locus. This approach has previously been used to locate the gene responsible for a large number of cases of CHARGE syndrome, which is also characterised by a number of congenital anomalies. A genome wide comparative genomic hybridisation profile suggested deletions in chromosomal band 8q12 in two affected individuals. The genes within this interval were sequenced and mutations in the gene *CHD7* were identified in 10 subjects {Vissers, van Ravenswaaij, et al 2004 192 /id}. However, a small deletion might still be missed by comparative genomic hybridisation depending on the resolution of the chips used.

6.7 Future work

The results of the genome scan of family R suggest that the most likely locus for the disease gene for hemifacial microsomia lies in the region 2q32 – 2q37. However, further investigation of the other chromosomal regions of interest that were identified by the genome scan is required in order to identify the disease related gene locus with greater certainty and accuracy. This would include analysis of further microsatellite markers with a greater density across the regions 3q25, 5q34-5q35, 8p21-8p23, 11q25, 13q34, and 16p12-16p13 and the region. Further marker studies would also be of interest in the region 10q23 – 10q24 that was sparsely covered in the scan, and within which the candidate gene *Fgf8* lies. A replacement for the failed marker D11S2019 would allow assessment of the region 11q12-11q13, a previously proposed locus for the disease gene.

Candidate genes within the strongest candidate region could then be identified, by consideration of the known expression patterns or function of the genes within that region or from the phenotype of animal models. These candidate genes could then be sequenced in order to identify mutations in affected individuals.

Identification of a gene, the mutation of which is responsible for the development of hemifacial microsomia, would help to characterise the phenotypic spectrum of the condition. Family members of affected individuals could be screened for mutations and counselled appropriately. Sporadic cases could be screened for mutation in an identified

disease gene, and this would provide information on the degree to which sporadic hemifacial microsomia is genetically determined.

This work has added to the epidemiological and phenotypic data previously available on hemifacial microsomia. Whilst the genome scan of family R did not identify a specific candidate locus, linkage to large sections of the genome was excluded, and the data have suggested defined regions of the genome that warrant further investigation. Future work directed by the results of this work should lead to further insights into the genetic basis of hemifacial microsomia.

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PROJECT: GENETIC BASIS OF MICROTIA

Patient information

1. Aim of the study:
To determine the genetic basis of the condition called microtia, where the individuals affected have a small or deformed ear. This condition can be bilateral and rarely may affect more than one member of the same family

2. Why is this study being done?
Microtia is a condition not fully understood in terms of how or why it occurs. It can be associated with other facial anomalies and may just represent one grade of a large spectrum. Most cases occur as an isolated finding in a single individual within a family, but occasionally more than one family member may be affected, suggesting a genetic predisposition in same families. We are aiming to find genetic factors which may predispose or protect against the development if microtia/hemifacial microsomia.

3. How is this study being done?
First we will inform the parents about the study before they attend their ENT or dental/maxillofacial clinic appointments. When they attend they will be given the option to participate in the study. This will involve answering some questions and taking blood samples from the child and the parents and occasionally of other relatives if they are also affected by the condition. In a few cases it may be necessary to visit the patient and his/her parents at home if the appointment is not due for a long time.

4. What are the risks and discomforts?
There are no anticipated risks and the only discomfort may arise from taking the required blood sample.

5. Who will have access to the case/research records?
Your participation will be completely confidential. Any identifying information will be removed before the information is entered in the computers. Your hospital files and photographic records will be seen by hospital staff only and will not be taken out of the hospital.

6. What are the potential benefits?
This study aims to further the understanding of the genetic involvement in the condition, but no results will be available to individuals since it is unlikely to benefit them directly for the foreseeable future.

7. Do you have to take part in the study?
If you decide, now or at a later stage, that you do not wish to participate in this research project, that is entirely your right, and will in no way prejudice any present or future treatment.

8. Who do you speak to if problems arise?
If you have any complaints about the way in which this research project has been, or is being conducted, please, in the first instance, discuss them with the researcher. If the problems are not resolved, or you wish to comment in any other way, please contact the Chairman of the Research Ethics Committee, by Post via the Research and Development Office, Institute of Child Health, , or if urgent, by telephone on ext. , and the Committee administration will put you in contact with him.

9. What are the arrangements for compensation?
This project has been approved by an independent research ethics committee who believes that it is of minimal risk to you. However, research can carry unforeseen risks and we want you to be informed of your rights in the unlikely event that any harm should occur as a result of taking part in this study.

No special compensation arrangements have been made for this project but you have the right to claim damages in a court of law. This would require you to prove fault on the part of the Hospital and/or any manufacturer involved.

10. Researcher who will have contact with the family:
You will be contacted by Mr Jerome Lim, Research Fellow in ENT or Dr. Maria Bitner-Glindzicz, Clinical and Molecular Geneticist.
Contact numbers:

- Mr Jerome Lim Ext. or page him via switchboard on the same number.
- Dr. M. Bitner-Glindzicz Ext.

PROJECT: GENETIC BASIS OF MICROTIA

Information for Parents

We would like to invite your child to participate in a study that looks at what causes some children to be born with deformed ears.

1. Aim of the study:
To determine the genetic basis of the condition called microtia, where the individuals affected have a small or deformed ear. This condition can be bilateral and rarely may affect more than one member of the same family

2. Why is this study being done?
Microtia is a condition not fully understood in terms of how or why it occurs. It can be associated with other facial anomalies and may just represent one grade of a large spectrum. Most cases occur as an isolated finding in a single individual within a family, but occasionally more than one family member may be affected, suggesting a genetic predisposition in same families. We are aiming to find genetic factors which may predispose or protect against the development if microtia/hemifacial microsomia.

3. How is this study being done?
First we will inform you about the study before you attend the ENT or dental/maxillofacial clinic appointments. When you attend you will be given the option to participate in the study. This will involve answering some questions and taking blood samples from you and your child and occasionally of other relatives if they are also affected by the condition. In a few cases it may be necessary to visit the patient and his/her parents at home if the appointment is not due for a long time.

4. What are the risks and discomforts?
There are no anticipated risks and the only discomfort may arise from taking the required blood sample.

5. Who will have access to the case/research records?
Your participation will be completely confidential. Any identifying information will be removed before the information is entered in the computers. Your hospital files and photographic records will be seen by hospital staff only and will not be taken out of the hospital. The research records may be reviewed by regulatory authorities. Your GP will be contacted if you wish to participate in the study.

6. What are the potential benefits?

This study aims to further the understanding of the genetic involvement in the condition and although the study will not be of direct benefit to the patients participating in it, we are hoping that the results obtained will benefit future patients.

7. Do you have to take part in the study?
If you decide, now or at a later stage, that you do not wish to participate in this research project, that is entirely your right, and will in no way prejudice any present or future treatment.

8. Who do you speak to if problems arise?
If you have any complaints about the way in which this research project has been, or is being conducted, please, in the first instance, discuss them with the researcher. If the problems are not resolved, or you wish to comment in any other way, please contact the Chairman of the Research Ethics Committee, by Post via the Research and Development Office, Institute of Child Health, , or if urgent, by telephone on ext. , and the Committee administration will put you in contact with him.

9. What are the arrangements for compensation?
This project has been approved by an independent research ethics committee who believes that it is of minimal risk to you. However, research can carry unforeseen risks and we want you to be informed of your rights in the unlikely event that any harm should occur as a result of taking part in this study.

No special compensation arrangements have been made for this project but you have the right to claim damages in a court of law. This would require you to prove fault on the part of the Hospital and/or any manufacturer involved.

10. Researcher who will have contact with the family:
You will be contacted by Mr Jerome Lim, Research Fellow in ENT or Dr. Maria Bitner-Glindzicz, Clinical and Molecular Geneticist.
Contact numbers:

- Mr Jerome Lim Ext. or page him via switchboard on the same number.
- Dr. M. Bitner-Glindzicz Ext.

Great Ormond Street Hospital for Children NHS Trust and Institute of Child Health Research Ethics Committee

Consent Form for PARENTS OR GUARDIANS of Children Participating in Research Studies

Title: Genetic basis of microtia/hemifacial microsomia.

NOTES FOR PARENTS OR GUARDIANS

1. Your child has been asked to take part in a research study. The person organising that study is responsible for explaining the project to you before you give consent.

2. Please ask the researcher any questions you may have about this project, before you decide whether you wish to participate.

3. If you decide, now or at any other stage, that you do not wish your child to participate in the research project, that is entirely your right, and if your child is a patient it will not in any way prejudice any present or future treatment.

4. You will be given an information sheet which describes the research project. This information sheet is for you to keep and refer to. ***Please read it carefully.***

5. If you have any complaints about the way in which this research project has been or is being conducted, please, in the first instance, discuss them with the researcher. If the problems are not resolved, or you wish to comment in any other way, please contact the Chairman of the Research Ethics Committee, by post via The Research and Development Office, Institute of Child Health, 30 Guilford Street, London WCIN IEH or if urgent, by telephone on 020 7242 9789 ext 2620 and the committee administration will put you in contact with him.

CONSENT

I/We _____ being the parent(s)/guardian(s) of _____

_____ agree that the Research Project named above has been explained to me to my/our satisfaction, and I/We give permission for our child to take part in this study. I/We have read both the notes written above and the Information Sheet provided, and understand what the research study involves.

SIGNED (Parent (s)/Guardian (s)) **DATE**

_____ _____

SIGNED (Researcher) **DATE**

_____ _____

Great Ormond Street Hospital for Children NHS Trust and Institute of Child Health Research Ethics Committee

Assent Form for CHILDREN Participating in Research Studies

Title: Genetic basis of microtia/hemifacial microsomia.

NOTES FOR CHILDREN

1. You have been asked to take part in some research. The person organising that study must explain the project to you before you agree to take part.

2. Please ask the researcher any questions you like about this project, before you decide whether to join in.

3. If you decide, now or at any other time, that you do not wish to be involved in the research project, just tell us and we will stop the research. If you are a patient your treatment will carry on as it would normally.

4. You will be given an information sheet which describes the research. This information is for you to keep and refer to at any time. *Please read it carefully.*

5. If you have any complaints about the research project, discuss them with the researcher. If the problems are not resolved, or you wish to comment in any other way, please contact the Chairman of the Research Ethics Committee, by post via The Research and Development Office, Institute of Child Health or if urgent, by telephone on ext and the committee administration will put you in contact with him.

ASSENT

I _____ agree that the Research Project named above has been explained to me to my satisfaction, and I agree to take part in this study. I have read both the notes written above and the Information Sheet about the project, and understand what the research study involves.

SIGNED _____ DATE _____

SIGNED (Researcher) _____ DATE _____

HFM - MICROTIA

SURNAME NAME

DOB HOSP. #

ADDRESS

POSTCODE

TEL. HOME

TEL. WORK

GP

ADDRESS

POSTCODE

TEL:

BLOOD SAMPLES	DOB	ECACC	DNA
PROBAND		☐	☐
FATHER		☐	☐
MOTHER		☐	☐
OTHER		☐	☐
		☐	☐
		☐	☐

MEDICAL HISTORY

Pregnancy

Bleeding
When How long Times

Medical problems
DM HT Other

Multiple pregnancy

Medication Alcohol Smoking

Delivery
Weeks Type Problems

Family History

Pregnancies (including proband) Bleeding

Problems Tags/pits

Other medical

Parents
Medical Hx.

Tags/pits

Grand-parents
Medical Hx.

Tags/pits

Uncles/Aunts
Medical Hx.

Tags/pits

Cousins
Medical Hx.

Tags/pits

PROBAND

Head Plagiocephaly yes/no Hydrocephalus yes/no

Neck

Face

Mandibular hypoplasia Maxillary hypoplasia

Cleft Lip Palate Midline Lateral

Facial palsy

Ears

Microtia Side Grade

Tags Pits

EAM

HL

Eyes

Micropthalmia Epibulbar dermoid

Mouth

Macrostomia

Mental development

Other Cardiac Renal

Syndrome?

Summary ideogram displaying multipoint lod score plots and their chromosomal relationships. Excursion of the plot to the left of the midline represents a positive lod score, and to the right of the midline indicates a negative lod score.

Addendum